Colonic Dysmotility in Children

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Dedication:

This thesis is dedicated to my wife, Nicola, and to my mother and father, Esther and Graham.

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Statement of conjoint work:

I personally performed all of the research contained in this thesis, with the following exceptions:

Section 3 - The scintigraphic images were performed and interpreted by Dr. David Cook (Consultant Radiologist, Department of Nuclear Medicine, Royal Children's Hospital, Melbourne). Data on the 101 patients was collected by Mr. Benjamin Cook (Department of Surgical research, Royal Children's Hospital). All of the text, including data analysis, the literature review, figure preparation and the discussion were undertaken by myself.

Chapter 4 - The first 24 (out of 74) muscle strip preparations described were carried out by Dr. Patricia Hengel (Department of Surgical Research, Royal Children's Hospital, Melbourne), using the same equipment and experimental technique. The immunofluorescence histochemistry for the preparations in Section 4 was undertaken by Mrs Pamela Farmer and Dr. Bridget Southwell (Department of Surgical Research, Royal Children's Hospital, Melbourne). Confocal microscopy was performed by Dr. Bridget Southwell, Mrs Pamela Farmer and myself. Data analysis of the immunohistochemistry studies was carried out by Dr. Bridget Southwell. Therefore, the methods, results and figures concerning the immunohistochemistry for this experiment have been included as Appendix 2 (section 10.2). Data analysis of the physiology studies, the literature review, figure preparation and the discussion in section 4 were performed by myself. Statistical analysis assistance was provided by Professor John Carlin and Miss Gabrielle Davies (Department of Biostatistics, Royal Children's Hospital, Melbourne).

Ethical Considerations:

The experimental protocols for the studies described in sections 4, 5 and 7 were approved by the Ethics-in-Human-Research Committee at the Royal Children's Hospital, Melbourne - reference numbers 98072B and 21049A respectively. The ethical approval for the *in vitro* studies of immunohistochemistry and neuromuscular transmission were granted on the basis that preliminary studies of colonic resections for intractable constipation had shown neuronal abnormalities and that previous studies (from other centres) had demonstrated neuronal anomalies in adults with slow-transit constipation. Ethical approval was granted for the study of colonic manometry as the catheter-insertion was likely to be painless for the children who volunteered and that the 24 hour studies would not produce any significant adverse-effects.

It would have been unethical to study healthy children to use as 'controls' for any of the studies described. Therefore, a meta-analysis of previous reports of gastro-intestinal transit in healthy children was used to define normal ranges of transit to compare with the scintigraphic studies carried out in children with intractable constipation. In addition, it was decided that it would not be possible, because of the risk of excessive radiation, to study two different methods of measuring gastro-intestinal transit (i.e. radio-opaque markers and scintigraphy) in the same children. Adult colon specimens were used as the best available tissue for comparison for the *in vitro* studies. A study of healthy young adults (using naso-colonic catheter insertion) performed under similar conditions was used to compare the findings of our paediatric manometry study.

Published work contained in this thesis (see also statement of conjoint work):

1. Cook BJ, Lim E, Cook D, Hughes J, Chow CW, Stanton MP, Bidarkar SS, Southwell BR, Hutson JM. Radionuclear transit to assess sites of delay in large bowel transit in children with chronic idiopathic constipation. Journal of Pediatric Surgery 2005; 40:478-483.
2. Stanton MP, Hengel PT, Southwell BR, Chow CW, Keck J, Hutson JM, Bornstein JC. Cholinergic transmission to colonic circular muscle of children with slow-transit constipation is unimpaired, but transmission via NK2 receptors is lacking. Neurogastroenterology and Motility 2003. 15(6): 669-78.
3. Stanton MP, Shin YM, Hutson JM. Laparoscopic placement of the Chait cecostomy device via appendicostomy. Journal of Pediatric Surgery 2002; 37(12): 1766-7.
4. Stanton M, Hutson JM, Simpson D, Catto-Smith A, Oliver M, Dinning P, Southwell B and Hutson JM. Colonic manometry via appendicostomy shows reduced frequency, amplitude and length of propagating sequences in children with slow-transit constipation. Journal of Pediatric Surgery 2005, *in press*.

Related Publications:

1. Marshall JM, Huston JM, Anticich N, Stanton MP. Antegrade continence enemas in the treatment of slow-transit constipation. Journal of Pediatric Surgery 2001; 36:1227-1230.
2. Shin YM, Southwell BR, Stanton MP, Hutson JM. Signs and symptoms of slow-transit constipation versus functional retention. Journal of Pediatric Surgery 2002; 37:1762-1765.

Presentations at International Conferences:

1. Cholinergic neurotransmission in the transverse colonic circular muscle of children with slow-transit constipation. Stanton MP, Hengel P, Bornstein JC, Hutson JM, Keck J, Southwell BR. Short oral/poster presentation at the American Academy of Paediatrics, Section on Surgery, San Francisco, October 19-21, 2001.
2. Laparoscopic placement of the Chait cecostomy device via appendicostomy. Stanton MP, Shin Y-M, Hutson JM. Oral presentation at the Pacific Association of Paediatric Surgeons, La Jolla, California, May 2002.
3. 24 hour colonic manometry shows reduced frequency, amplitude and length of propagating sequences in children with slow-transit constipation. Stanton MP, Hutson JM, Oliver MR, Simpson D, Dinning P, Southwell BR and Catto-Smith A. Oral presentation at the British Association of Paediatric Surgeons Annual Conference, Estoril, Portugal, July 2003.

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Abstract:

The literature review of this thesis describes the development, structure and function of the enteric nervous system and the known congenital anomalies, which can cause colonic dysmotility. The clinical investigations used to assess children with idiopathic chronic constipation are outlined, together with the medical and surgical therapies used in the treatment of this condition. Four studies are reported, the first of which is a retrospective review of 101 scintigraphic studies of colonic transit in the investigation of children with idiopathic chronic constipation. Second, an *in vitro* study of neuromuscular transmission in colonic circular muscle from adults (with carcinoma) and children with slow colonic transit (defined on radio-isotope study) is described. Third, a retrospective review of the clinical outcome of 11 children with severe slow-transit constipation in whom laparoscopic formation of an appendicostomy for colonic washouts has been carried out is reported. The fourth study is series of 7 children in whom per-appendicostomy insertion of a catheter and subsequent 24-hour studies of multipoint colonic manometry was undertaken. The results of these studies are then discussed to explain the possible implications in relation to the knowledge of the aetiology, clinical investigation and management of children with colonic dysmotility. The overall hypothesis of this thesis is that children with idiopathic chronic constipation have identifiable physiological abnormalities in colonic motor function, which may be due to enteric neuronal abnormalities.

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indicate the number of specimens tested; note this was less than the total sample for technical reasons. Contractions induced by EFS are significantly reduced by incubation with hyoscine in all types of CCM. Column labels are as for Figure 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, comparing EFS pre and post hyoscine, paired t test).

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Table Legends:

Table 1: Results of 9 studies of gastro-intestinal transit in healthy children.

Two types of study have been reported – using carmine dye or using radio-opaque markers (ROM). Studies reported either total gastro-intestinal transit time (TGITT) or colonic transit time (CTT) (both in hours). Transit times (in hours) through the right colon, the left colon and the recto-sigmoid are shown where these were reported. The mean total transit time (in hours) and upper limit of normal (ULN) is also shown. The upper limit of normal was either reported directly or calculated as the mean plus 2 standard deviations.

Table 2: Geometric centres of radioactivity in 101 children with chronic constipation.

The means, standard deviations (SD) and ranges of the geometric centres (GC) of radioactivity at 6 hours, 24 hours, 30 hours, 48 hours and the GC sum are shown. Patients have been classified into subgroups using visual interpretation of acquired images into those with normal colonic transit, functional faecal retention and slow colonic transit.

Table 3. Ages (mean standard deviation, SD and range) of patients and number (N) of samples of transverse and sigmoid colon from adults and from children with slow-transit constipation.

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Abbreviations used (in the order that they appear in the text):

STC – Slow transit constipation

ENS – Enteric nervous system

ICC – Interstitial cells of Cajal

Ach – Acetylcholine

TK – Tachykinin

CCM – Colonic circular muscle

ChAT – Choline Acetyltransferase

NO – nitric oxide

NOS – nitric oxide synthase

SP – substance P

NK – neurokinin

NKA – neurokinin A

NKB – neurokinin B

NANC – non-adrenergic non-cholinergic

VIP - vasoactive intestinal peptide

ATP – adenosine 5'-triphosphate

PACAP – pituitary adenylated cyclase-activated peptide

cGMP – cyclic guanosine monophosphate

NADPH – nicotinamide-adenine dinucleotide phosphate diaphorase

L-NNA - N^G-nitro-L-arginine

L-NAME - L- N^G-nitro arginine methyl ester

EFS – Electrical field stimulation

NOLA - Nitro-L-arginine

MMC – Migrating motor complex

CNS – Central nervous system

HD – Hirschsprung Disease

GDNF – Glial cell-line derived neurotrophic factor

EDNRB - Endothelin-B receptor

CIPO – Chronic intestinal pseudo-obstruction

SMP – Submucous plexus

MP – Myenteric plexus

IND – Intestinal neuronal dysplasia

GC – Geometric centre

ROI – Region of interest

ROM – Radio-opaque marker

TGITT – Total gastro-intestinal transit time

CTT – Colonic transit time

ULN -Upper limit of normal

RAIR – Recto-anal inhibitory reflex

PS – Propagating sequences

HAPS – High amplitude propagating sequences

HAPC – High amplitude propagating contractions

PEG – Polyethylene glycol

ACE – Antegrade continence enema

SD – Standard deviation

FFR -Functional faecal retention

DMSO – Dimethylsulphoxide

PBS - Phosphate buffered saline

SP-IR – Substance P immunoreactivity

TK-IR – Tachykinin immunoreactivity

TTX – Tetrodotoxin

SEM – Standard error of the mean

ANOVA – Analysis of variance

SNP - Sodium nitroprusside

APS – Antegrade propagating sequence

SH – Sidehole

RPS - Retrograde propagating sequence

AUC – Area under the curve

R - Region

1. Introduction:

Constipation is a very common condition in childhood. Most cases follow a painful episode of stool evacuation and the child responds to this with 'stool withholding' behaviour to prevent this recurring. This condition is usually self-limiting, or responds to simple medical treatments, such as dietary modifications or laxatives[2-5]. However, in a proportion of children, the constipation becomes intractable and may be associated with more severe symptoms including bloating, vomiting, soiling (secondary to long-term rectal distension) and failure to thrive[6, 7]. In these cases, behaviour, social interactions, education and family dynamics can be significantly disturbed[8].

Chronic intractable constipation in childhood is a poorly characterised condition in which the aetiology is unknown and there are no universally accepted diagnostic criteria or treatment strategies. Affected children are often labelled as having 'idiopathic chronic constipation'. Since many children with chronic constipation are subsequently referred to paediatric gastroenterology outpatient clinics, the health expenditure associated with this condition is considerable[2]. The significance of this problem is highlighted by the recommendations of the Research Agenda for Paediatric Gastroenterology in a report published in 2002 by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition[9].

The clinical investigation of children with idiopathic chronic constipation is still somewhat rudimentary. Studies of gut transit times, using ingested radio-opaque markers, are commonly employed to provide an assessment of the severity of constipation[10-13]. These studies are adapted from techniques described in the 1960s

where dyes or beads were ingested and then collected in the stool to give an estimate of total gut transit time[14-16]. There are no universally agreed normal values for gut transit times in children, partly due to the ethical difficulties in studying healthy children. This compounds the problem of effectively evaluating and classifying this group of children and, therefore, in investigating possible aetiological factors.

Therefore, as part of the review of the medical literature in this study, a meta-analysis of available reports of gut transit times in healthy children has been undertaken to provide a basis for the classification of colonic motility disorders. The radiological techniques for investigating gastro-intestinal transit in adults were also reviewed in order to assess the potential for applying an alternative radiological method of investigating and classifying colonic dysmotility in children. Radio-opaque marker studies are widely used to investigate children with colonic dysmotility[10, 11, 13]. However, in adults, radioisotope studies (scintigraphy) are often employed to assess chronic constipation[17-19]. Slow-transit constipation (STC) has become a well-recognised disease entity in adults in which nearly all affected patients are female[20, 21].

The initial study in this thesis is a retrospective review of 101 consecutive radio-isotope studies carried out on children with idiopathic chronic constipation. The aim was to determine the validity of this investigation in sub-classifying this heterogeneous group and to assess whether any of these children have identifiable slow colonic transit.

Even when colonic transit can be defined in this group of children, the question often arises as to whether children with evidence of slow colonic transit really have an identifiable, possibly congenital, defect in the motility of the colon that causes their symptoms, or whether the initial behavioural disturbances can become chronic and cause severe secondary gastro-intestinal symptoms. In order to answer this question, it is first necessary to understand how the motility of the colon in humans is controlled under normal conditions and then determine whether children with delayed colonic transit demonstrate abnormal colonic motor characteristics.

It has been recognised since 1899 that the gut has its own intrinsic nerve supply, called the enteric nervous system, which can function independently of extrinsic control[22-24]. The central nervous system exerts a mainly modulatory influence over the enteric nervous system. The basic reflex of the enteric nervous system is peristalsis, where a wave of proximal gut smooth muscle contraction is co-ordinated with distal relaxation to allow antegrade passage of a bolus of intestinal content. It is currently unclear if children with idiopathic chronic constipation have a physical defect in the machinery of peristalsis, i.e. in the nerves or muscle of the gut. An alternative explanation might be that the central nervous system, via learned behaviours such as stool withholding, can exert sufficient influence over the enteric nervous system to cause the symptoms associated with idiopathic chronic constipation.

In order to address this issue, the normal development, anatomy and function of the human enteric nervous system has been reviewed in this thesis (section 2.1-2.3). The circuitry of the enteric nervous system in, for example, the guinea-pig small intestine,

is now well understood[25, 26]. However, the findings in animal species have not been correlated with the human enteric nervous system, partly due to the inherent problems with obtaining human tissue for study. It can be seen that it is necessary for the function of the normal human enteric nervous system to be further evaluated to provide a basis for the investigation of motility disorders.

The available literature concerning the aetiology and management of the known congenital enteric nervous system anomalies causing colonic dysmotility in humans, (Hirschsprung disease, intestinal neuronal dysplasia and hypoganglionosis) has also been assessed, to determine the validity and reliability of these diagnoses (section 2.5). While Hirschsprung disease is well defined, the diagnostic criteria for the latter two diagnoses are highly controversial[27, 28]. In light of the advances in the understanding of the structure and function of the enteric nervous system, alternative hypotheses for the causes of non-Hirschsprung cases of colonic dysmotility can be investigated.

As the circuitry of the enteric nervous system is contained within the wall of the gut, small strips of muscle can be studied *in vitro* in order to investigate neuromuscular function[29, 30]. For part of the research for this dissertation, specimens of colonic muscle from adults (with carcinoma) and from children with defined slow colonic transit have been used to study neuromuscular transmission. Isotonic responses to drug agonists and to electrical field stimulation were measured in order to investigate the properties of 'normal' adult tissue and tissue from children with slow colonic transit. Low nerve fibre density of tachykinin (a family of neurotransmitters) has been reported previously in both adults and children with slow-transit constipation using

immunofluorescence histochemistry[6, 31]. Therefore, colonic muscle specimens from children with both normal and low tachykinin nerve fibre density were compared, to assess the functional significance of this histological finding.

The poor understanding of the aetiology of motility disorders in children and the lack of definitive diagnostic criteria mean that treatment strategies are necessarily empirical in nature. Medical therapies include oral administration of bulking agents and stimulant laxatives[2, 4, 5]. Rectal enemas are often used to treat rectal impaction, but this approach is considered to be unnecessarily invasive by some[32]. Surgical interventions for extreme cases involve excising part, or all, of the dysmotile colon[33, 34]. As serious complications can occur following such colonic surgery, less invasive surgical strategies have been developed, principally the 'antegrade continence enema'[35]. This involves using the appendix as a conduit between the skin and the colon, through which colonic washouts can be directed. The antegrade continence enema is now used worldwide for the management of various colonic motility disorders[36-38]. Ethical considerations mean that entirely new surgical treatments are rarely developed and it is more appropriate that modifications of existing therapies are assessed. Thus, in this series of studies, the efficacy of combining a minimally invasive surgical technique to form an appendix stoma with the use of a low-profile design of appendicostomy catheter has been retrospectively reviewed.

Manometry refers to the measurement of the *in vivo* pressure characteristics within the lumen of the gastro-intestinal tract. Manometry of the anorectum, or of part of the colon, has been employed in relation to chronic constipation in children, mainly as a

research tool[39-41]. The technique currently has limited applicability as a clinical investigation. However, the development of colonic manometry has allowed more sophisticated and prolonged studies of colonic physiology to be undertaken in healthy adults[1, 42]. The pressure characteristics associated with diurnal variation, the response to eating and the response to waking have been described. However, 24-hour manometric studies of the whole colon have not been attempted in children because of the difficulties of catheter placement. Manometry is potentially a valuable area of research with respect to colonic motility disorders in children[43, 44]. It may allow definitive assessment of *in vivo* physiology and so answer the question of whether learned behaviours controlled by the central nervous system or a physical defect in the automatic function of the enteric nervous system underlie colonic dysmotility in children. In this study, a new method of colonic manometry catheter placement has been tested and the *in vivo* colonic motor characteristics of 6 children with scintigraphic slow colonic transit have been measured.

2.1 Overview of the enteric nervous system:

The gross structure of the colon is similar to that of the rest of the gastro-intestinal tract and so its layers consist of mucosa (epithelial lining, connective tissue and muscularis mucosae), submucosa, muscle wall and serosa. The submucosa and serosa both contain blood vessels, lymphatics and nerves. The muscle wall is composed of smooth muscle cells and these are arranged in 2 perpendicular layers – the outer longitudinal layer and the thicker inner circular layer. In the human colon, the longitudinal layer is concentrated into 3 separate bands known as the taeniae coli[45]. The functions of the human colon are propulsion of intestinal content, storage of faeces and absorption of water and electrolytes[46]. The basic unit of propulsive action of the gut is the peristaltic reflex, which is controlled by the enteric nervous system. The peristaltic reflex of the gastro-intestinal tract was first described by Bayliss and Starling in the 19th century[22-24] and was named the 'Law of the Intestine'. This reflex consists of descending inhibition and ascending contraction to allow passage of a bolus.

The enteric nervous system (ENS) is a branch of the autonomic nervous system and consists of plexuses of ganglia, which are located within the wall of the gut. The total number of neurones of the ENS has been estimated to be between 10 and 100 million, a total similar to that of the spinal cord[26]. It has been recognised since 1899 that the ENS can function independently of the rest of the autonomic nervous system[22-24]. The central nervous system has a modulatory influence over the ENS.

The plexuses of the ENS are chiefly concentrated in the submucosal layer and between the outer longitudinal and the inner circular muscle layers. The location of

the submucosal plexuses was first described by Meissner in 1857 and that of the myenteric plexuses by Auerbach in 1862[25, 26].

The neurones that mediate this sensory-motor function of the gut are sensory primary afferent neurones, connecting interneurones and motor neurones to smooth muscle cells[47]. The functional classes of neurones in the ENS of the guinea-pig ileum have been fully described, but to a much lesser degree in the human ENS. Two classifications of neuronal types exist, based on studies of the guinea-pig ileal myenteric plexus. Electrophysiological studies have identified neurones with a long hyperpolarisation after action potentials (AH neurones) and neurones that display fast excitatory post-synaptic potentials (S neurones). Histochemical studies undertaken by Dogiel categorised neurones by their shape into 3 types – Dogiel type I monoaxonal neurones, Dogiel type II multiaxonal neurones and Dogiel type III filamentous neurones. S neurones show Dogiel type I and type III (but not type II) shapes, whereas AH neurones show Dogiel type II structure[25, 26, 48].

Primary afferent neurones react to chemical stimulation and to mechanical deformation such as radial stretch. Excitatory and inhibitory motor neurones innervate the circular and longitudinal muscle. Interneurones connect other neurones in both ascending and descending directions. In addition, the myenteric ganglia contain secretomotor and vasomotor neurones. The neurotransmitters associated with the enteric neurones include acetylcholine, the tachykinin peptides, nitric oxide, vasoactive intestinal peptide and adenosine triphosphate[25, 26, 47, 48].

The enteric motor neurones act on smooth muscle cells to elicit contraction or relaxation, on blood vessels, on secretory cells/glands and on interstitial cells of Cajal (ICCs). The ICCs have been shown to have a role as pacemaker cells as well as in propagating electrical activity and mediating neurotransmission[49-53]. The enteric smooth muscle cells, under the influence of ICCs, generate oscillations known as slow waves in their resting membrane potentials (the electrical difference between the interior and exterior of the cell). These oscillations in turn provoke action potentials and thus smooth muscle contraction/relaxation. Smooth muscle activity is under myogenic, neurogenic and chemical control[54, 55]. In the colon, smooth muscle contractions are organised into 3 recognisable types – individual phasic contractions, organised groups of contractions and special propulsive contractions[54].

2.2 Development of the enteric nervous system:

All of the neurons that make up the ENS are derived from the embryological neural crest. Cells originating from the vagal, truncal and sacral neural crest levels are known to colonise the gut of several species. This colonisation occurs by a process of migration, proliferation and differentiation[56-60].

The ectoderm of the developing embryo is recognisable as a distinct layer by the third week of embryogenesis, having formed (with the endoderm and mesoderm) by a process known as gastrulation. The ectoderm undergoes neuralation to form the neural crest. Cells of the neural crest are migratory and are subsequently distributed to form a range of tissues including neurones (of the peripheral and autonomic systems), glial support cells, melanocytes, the adrenal medulla and facial cartilage[61, 62]. Cells derived from the neural crest are not recognisable phenotypically as neuronal or glial

cells during the process of migration. However, the origin of gut neurones has been determined in certain species by either ablation or transplantation techniques[56, 57, 59, 60, 63].

Studies carried out in chick embryos showed that ablation of the neural crest at the level of somites 1-7 (vagal level) resulted in complete agenesis of the ENS along the whole length of the gut. Results of these original experiments suggested that neurones of the ENS were entirely derived from this level only and migrated via an oro-anal wave[56]. It is now known that some neurones of the ENS are derived from the lumbosacral level of the neural crest[59].

Transplantation techniques were reported in the 1970's, in which the sacral level of the neural crest was confirmed as a source of ENS neurones. These studies were conducted by removing the lumbosacral neural tube of chick embryos and transplanting the equivalent neural tube from quail embryos ('chick-quail transplantation'). The subsequent migration and differentiation of the transplanted cells were demonstrated using histological techniques[57]. Subsequent studies outlined whether sacral level neural crest cells differentiated into neurones or glial support cells, and ascertained the timing of migration. It is now assumed that (in birds) the majority of ENS neurones are of vagal level origin. Some hindgut neurones, particularly in the myenteric plexus, have a sacral level origin from the neural crest[59]. Migration is thought to occur by a combination of cell-to-cell contact and chemotaxis[60, 63].

There are two important and relatively well-defined signalling pathways that regulate migration, differentiation and development of the ENS – the RET/GDNF/GFRα1 and the EDN3/EDNB pathways. Glial cell derived neurotrophic factor (GDNF) is produced by fetal gut mesenchymal cells and forms a complex with the RET receptor and its co-receptor GFRα1. This receptor complex controls the migration and differentiation of vagal neural crest cells that subsequently colonise the entire fetal gut. RET expression is induced by transcription factors including SOX10 and PHOX2B. Endothelin-3 (EDN3) is a signalling ligand, which, with its receptor endothelin-B, controls hindgut development and differentiation. Mutations in these genes cause various degrees of ENS dysganglionosis in animal models and are also implicated in the aetiology of Hirschsprung disease in humans[60, 63-66].

2.3 Colonic motor function:

The movement of colonic content occurs due to a co-ordinated combination of smooth muscle excitation/contraction and inhibition/relaxation. This allows the antegrade passage of faecal matter. Various neurotransmitters have been proposed as having roles in mediating nerve-to-nerve and nerve-to-muscle transmission and thus in eliciting contraction and relaxation. The principal transmitters associated with contraction are acetylcholine and the tachykinins. Those thought to mediate relaxation include nitric oxide, vasoactive intestinal peptide and adenosine triphosphate.

2.3.1 Excitation and contraction:

Acetylcholine (Ach) and the tachykinins (TKs) are widely distributed within the ENS of mammals[25, 67]. The TKs are a group of peptides of which substance P, neurokinin A and neurokinin B are found in the ENS. It has been concluded that Ach and

tachykinins act as co-transmitters in mediating excitation/contraction in the guinea pig ileum and colon[68]. However, there remains some controversy with respect to the role of these neurotransmitters in human colon.

2.3.1.1 Histological studies of cholinergic innervation:

A combination of retrograde labelling and fluorescence immunohistochemistry has been used to identify the neurotransmitters associated with motor neurones in human colonic circular muscle (CCM)[69-72]. Ach is synthesised from choline by the enzyme choline acetyl transferase (ChAT). ChAT has been identified in just over 50% of all neurones projecting to the human CCM, with the remaining neurones projecting to the CCM being immunoreactive for the enzyme which synthesises nitric oxide, nitric oxide synthase (NOS). None of the NOS-immunoreactive neurones supplying the CCM was also immunoreactive for ChAT. The great majority (86%) of excitatory ACh-containing motor neurones project orally[71], while 77% NOS-immunoreactive neurones project anally. About 23% of the orally projecting neurones that supply the CCM are immunoreactive for TKs[72], suggesting that many of the ChAT-immunoreactive neurones supplying the CCM also contain TKs. ACh and TKs both cause smooth muscle excitation and NO causes it to relax, so it has been concluded that the ChAT-immunoreactive neurones are excitatory motor neurones, while the NOS-immunoreactive neurones are assumed to be inhibitory motor neurones.

2.3.1.2 Functional studies of cholinergic neurotransmission:

The enteric nervous system is embedded within the walls of the human gut and can function independently of the central nervous system. Therefore, reduced preparations of gut tissue can be used to study neuromuscular transmission[29, 73]. Functional

studies conducted in the 1960's and 1970's reported that ACh was likely to have a significant role in mediating excitation/contraction in the human colon[29, 73]. Small strips (1-2 mm x 15-30 mm) of colonic muscle (removed at cancer resection surgery) were used and isotonic contractions were demonstrated in response to electrical stimulation. Fishlock[73] showed that ACh elicited contractions and that these responses were blocked in the presence of the muscarinic antagonist atropine. Bennett and Stockley[29] reported subsequently that strips of colonic muscle contracted in response to electrical stimulation. Again these contractions were reduced, but not fully abolished, in the presence of cholinergic blockade (atropine and hyoscine). Hughes *et al*[74] compared cholinergic responses in colonic muscle with and without evidence of cancer. No differences between responses elicited by ACh were found between the two types of tissue.

Couture *et al*[75] have demonstrated that both the Ach analogue carbachol and substance P (one of the TKs) both elicit contractions in human colon[75]. This study also demonstrated that overnight storage of gut tissue did not affect the responses elicited. Kerr *et al*[76] investigated the subtypes of muscarinic receptors (M1-M6) that mediate cholinergic transmission in human colon. It was concluded that the M_3 subtype was activated predominantly. Regional variation in ACh sensitivity in the human colon has been reported, with a higher sensitivity to carbachol occurring in the ascending colon compared to the sigmoid colon.

In recent years there has been much interest in the functional role of tachykinins in mediating excitatory neuromuscular transmission. Tachykinins are known to co-localise with ACh and so it is likely that both act as co-transmitters, with ACh in

mediating excitation/contraction in both the guinea pig ileum and distal colon. Similarly, ChAT-immunoreactive and TK-immunoreactive neurones are co-localised in human CCM. A recent study by Cao *et al*[30] concluded that excitation/contraction in the human sigmoid CCM was entirely mediated by TKs acting via NK_2 receptors. As contractions were not reduced following incubation with atropine it was concluded that there was no cholinergic component to excitation/contraction. This result conflicts significantly with the findings of Stockley and Bennett[29].

Functional cholinergic neurotransmission has been described in relation to adult STC, but not in childhood idiopathic chronic constipation. Slater *et al*[77] reported that larger contractile responses were elicited by carbachol in CCM from adults with STC compared to controls. It was proposed that a 'denervation hypersensitivity' state might occur in response to impaired cholinergic neurotransmission. A possible functional deficit in cholinergic neurotransmission was also suggested by Burleigh *et al*[78], who demonstrated a decrease in the amount of radiolabelled ACh released when electrical field stimulation was applied to strips of colonic longitudinal muscle from adults with STC. An impaired manometric response to cholinergic stimulation (elicited using intravenous edrophonium) has been demonstrated in the descending colon of adults with STC[79]. However, Mitolo-Chieppa *et al*[80] reported that atropine reduced the EFS-induced frequency-tension curves by the same degree in STC adult colon and controls. Therefore, it was concluded that there was no abnormality in cholinergic innervation in adult STC. Cholinergic neurotransmission has not been studied previously in children with idiopathic chronic constipation.

2.3.1.3 Tachykinins

The TKs are a family of peptides found within both the enteric and central nervous systems. Three tachykinins have been identified in the enteric nervous systems of mammals – substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). The common structure found in these 3 peptides is the C-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. Three tachykinin receptors have been defined – NK_1, NK_2 and NK_3 receptors. These receptors have different affinities for the tachykinin peptides, with SP having the greatest affinity for NK_1 receptors, NKA having the greatest affinity for NK_2 receptors and NKB having greatest affinity for NK_3 receptors[67, 81].

The locations of tachykinin receptors have been defined in guinea pig gut. NK_1 receptors have been demonstrated on excitatory neurones, inhibitory neurones and inhibitory interneurones in the guinea pig ileum[82, 83]. In the guinea pig colon, NK_1 receptors have been identified on inhibitory motor neurones[83]. NK_2 receptors are located on smooth muscle cells[84] and NK_3 receptors have been shown on neurones only[85]. As outlined in the previous section on ACh, 23% of orally projecting neurones in human colonic circular muscle are immunoreactive for tachykinins and so many ChAT immunoreactive motor neurones co-localise tachykinins.

2.3.1.4 Functional studies of tachykininergic neurotransmission:

Tachykinin agonists and non-peptide tachykinin receptor antagonists have been developed and investigations of neuromuscular transmission have been conducted using similar experimental conditions to those used to study cholinergic pathways. Neurokinin-A (4-10) has been shown to be selective a NK_2 receptor agonist[86].

Examples of TK antagonists include the non-peptide SR 140333, which has been shown to act as a NK_1 receptor antagonist[87]; SR 48968, which blocks NK_2 receptors[88] and SR 142801, which antagonises NK_3 receptors[89].

There is increasing evidence that it is the NK_2 receptor subtype that plays the dominant role in mediating tachykininergic excitatory neurotransmission. Giuliani *et al*[90] and Croci *et al*[88] have concluded that it is the NK_2 receptors, but not NK_1 or NK_3 receptors that influence CCM contractions. Warner *et al*[91] also confirmed that NKA binds predominantly to NK_2 receptors to mediate human circular muscle contraction, and again, no significant role for NK_1 receptors was defined.

This concept of NK_2 receptor mediated tachykininergic circular muscle contraction has been extended recently. Cao *et al*[30] reported that excitation/contraction in human sigmoid CCM was entirely mediated by NKA acting via NK_2 receptors. It was reported that incubation with atropine did not alter contractions and so there was no role for ACh in mediating neuromuscular transmission. This finding conflicted with those of Bennett and Stockley[29]. Possible explanations for this disparity are that Cao *et al* measured isometric rather than isotonic contractions and used a shorter incubation time (15 minutes) with atropine.

Impaired colonic tachykininergic neurotransmission has been reported in studies of adults with STC. Menzies *et al*[92] reported that NKA-induced contractions were increased in colonic muscle preparations from adults with STC compared to controls. It was suggested that this phenomenon could be explained by an increase in NK_2

receptor numbers secondary to reduced tachykinin levels. Mitolo-Chieppa *et al*[93] described that CCM from adults with STC was hyporesponsive to NK_2 and NK_3 agonists, compared to control tissue.

2.3.2 Inhibition and relaxation:

Bayliss and Starling first appreciated the phenomenon of distal relaxation of the circular muscle during antegrade propulsion of intestinal content in 1899[22]. The reflex consisting of ascending excitation/contraction and descending inhibition/relaxation was named the 'Law of the Intestine'. Subsequently, neurotransmitters have been investigated as potential mediators of inhibition/relaxation in the small intestine and in the colon. Bennett and Stockley identified in 1975 that relaxation in colonic smooth muscle could be elicited in response to electrical field stimulation by using different stimulatory parameters to those that elicit contraction[29]. As these relaxations are not reduced by guanethidine or α/β adrenergic receptor blockade and occurred in the presence of hyoscine, inhibition/relaxation in colonic smooth muscle has been referred to as 'non-adrenergic non-cholinergic' (NANC). Nitric oxide (NO), vasoactive intestinal peptide (VIP), adenosine 5'-triphosphate (ATP) and pituitary adenylated cyclase-activating peptide (PACAP) have been proposed as inhibitory NANC neurotransmitters in the gut of animal species and humans[75, 94-99].

Nitric oxide is synthesised from L-Arginine by the enzyme NO synthase (NOS) and is located in multiple organ systems including the vascular system and the central nervous system[100]. NO acts as a neurotransmitter by activating guanylate cyclase and this leads to a rise in intracellular cyclic guanosine monophosphate (cGMP).

Elevation of cGMP induces smooth muscle relaxation[100]. Identification of potential inhibitory neurotransmitters has, in turn, led to the mechanism of inhibition/relaxation being studied in colonic motility disorders.

2.3.2.1 Distribution of inhibitory motor neurones:

Immunoreactivity for NOS and VIP has been demonstrated in motor neurones in human CCM. Nicotinamide-adenine dinucleotide phosphate diaphorase (NADPH) is co-localised with NOS and so has also been used as a marker for neuronal NO in the gastro-intestinal tract. However, the degree of co-localisation is debated, with some authors suggesting that it is approximately 90%, which would suggest that NADPH might not be sufficiently specific for use as a marker for NOS[101]. ATP-immunoreactivity in motor neurones has not been reported in human CCM.

VIP and NOS-positive neurones have been demonstrated throughout the human gastro-intestinal tract and their distribution has been shown to be higher in the right colon than the left colon[69, 102]. The populations of inhibitory neurones in the colon have been further defined using retrograde labelling and immunohistochemistry techniques. 45% of all CCM motor neurones are inhibitory, all of which are NOS-positive. Only a small proportion of NOS-positive neurones co-localise VIP and no inhibitory neurones contain VIP alone[71]. This has led to the conclusion that NO is the predominant neurotransmitter present on inhibitory motor neurones in human CCM, with VIP having a smaller role. In the submucous plexus, this ratio seems to be reversed, with VIP-positive neurones outnumbering NOS-positive neurones[103]. This is likely to reflect the role of VIP in secretomotor function.

2.3.2.2 Functional studies of inhibitory neurotransmission:

Evidence that NO ('nitrergic relaxation'), and possibly VIP ('peptidergic relaxation') or ATP ('purinergic relaxation'), mediate inhibition/relaxation in human CCM has come from contractility studies of muscle strips and intracellular electrophysiological recordings made using impalement of muscle cells. NOS antagonists such as N^G-nitro-L-arginine (L-NNA) and L- N^G-nitro arginine methyl ester (L-NAME), VIP antagonists (e.g. VIP 6-28) and ATP antagonists (e.g. apamin, a calcium-dependent potassium channel blocker) have been used to identify the components of functional inhibition/relaxation[80, 94-96].

Burleigh *et al*[94] demonstrated that NO at least partly contributes to isotonic relaxations of sigmoid CCM and internal anal sphincter muscle. NANC relaxations were elicited in response to electrical field stimulation (EFS). In the sigmoid CCM, such relaxations were partially reduced in the presence of L-NNA. L-NNA completely abolished relaxations in IAS muscle.

Boeckxstaens *et al*[95] have reported studies on both nitrergic and purinergic relaxation. Isometric NANC relaxations in proximal and distal CCM strips were measured in response to NO and in response to EFS. NO-induced relaxations were blocked by L-NNA. ATP induced smaller relaxations than those elicited by NO and these were blocked by apamin. EFS-induced relaxations were partially inhibited by L-NNA at lower frequencies and fully abolished at higher frequencies. L-NNA-resistant relaxations were further reduced in the presence of apamin. Thus, on the basis of these contractility studies, it appears likely that NO, possibly with ATP as a co-transmitter, mediates inhibition/relaxation in human CCM.

Further support for this concept of NO and ATP acting as co-transmitters was provided in a study which used both mechanical and electrophysiological techniques to investigate NANC relaxation in sigmoid CCM[96]. The inhibition of rhythmic contractions elicited by electrical field stimulation was reduced by both apamin and L-NAME. Electrophysiological recordings of impaled muscle cells demonstrated a fast hyperpolarisation inhibitory response evoked by a single stimulus, sensitive to apamin, but not L-NAME. A sustained hyperpolarisation response was evoked by repeated stimulation; this was reduced by both L-NAME and apamin.

VIP has been shown to mediate inhibition/relaxation in guinea pig, rabbit and dog intestine and to contribute to the descending relaxation component of human jejunal peristalsis[98]. In a study of the peristaltic reflex conducted on flat sheets of human jejunum, it was reported that release of VIP was increased during stretch-induced descending relaxation and that VIP antagonism caused inhibition of the relaxation response. VIP also reduced the size of contractions in human CCM evoked by acetylcholine and substance P[75]. However, other investigators have found no significant physiological role for VIP in mediating neuronal inhibition/relaxation[80].

There is some rather conflicting evidence regarding the role of NO in the regulation of overall transit of colonic content. Perfusion of Nitro-L-arginine (NOLA, NO antagonist) increased the frequency of migrating motor complexes (MMC) generated in mouse colon and increased the resting tone[104]. In rat colon, it has been reported that transit was impaired by NO blockade and, therefore, that NO promoted transit by enhancing relaxation[105]. As MMC are responsible for antegrade colonic content

propulsion, it is difficult to explain these apparently opposite effects of nitrergic inhibition.

2.3.2.3 Inhibitory innervation in colonic motility disorders:

That the 'spastic' colon seen in motility disorders, such as Hirschsprung disease and idiopathic chronic constipation, might be attributable to a failure of relaxation is an attractive theory. Much of the recent work concerning the aetiology of motility disorders has, therefore, focussed on potential histological abnormalities in densities of NO- or VIP-containing motor neurones, or in possible functional defects in nitrergic or peptidergic inhibitory neurotransmission.

Reduced expression for the neuronal NOS gene has been reported in the aganglionic colon of patients with Hirschsprung disease, compared to ganglionic colonic segments from the same patients[106]. Deficient NOS- and VIP-immunoreactivity has been demonstrated in aganglionic colon, both compared to the ganglionic colon of the same patients, and compared to 'control' colon[107, 108]. NOS- and VIP-immunoreactivity, however, was shown to be present in the extrinsic hypertrophic nerve bundles of the aganglionic segments[108]. It is not clear whether these defects in aganglionic colon equate to a deficiency in the NO-VIP axis, as proposed by Bealer *et al*[107], or whether this is merely secondary to the known absence of ganglion cells and intrinsic nerve fibres in Hirschsprung disease.

In vitro contractility studies have also suggested abnormalities in nitrergic and peptidergic inhibition/relaxation in the colon in Hirschsprung disease. NANC isometric, neuronal relaxations can be elicited in response to EFS in ganglionic, but

not aganglionic, colonic segments[109, 110]. In ganglionic colon, EFS-induced relaxations are inhibited by NO analogues (L-NAME or L-NNA), confirming that inhibition/relaxation is, in part at least, mediated by NO. Ciftci *et al*[111] reported that EFS-induced contractions increased in size in the presence of L-NNA in ganglionic, but not aganglionic colon. This implies that greater contractions were 'revealed' in the presence of blockade of inhibition/relaxation. However, it may be more appropriate to measure NANC relaxations directly, to ensure that responses measured are not due to an NO effect on cholinergic pathways.

Studies of nitrergic and peptidergic innervation in idiopathic chronic constipation have produced less consistent conclusions. Reduced VIP-IR has been reported in the descending colon from adults with idiopathic chronic constipation, compared to 'controls'[112]. NO antagonism increases isometric contractions in 'control' colon, but not in acquired megacolon (secondary to chronic constipation)[113]. However, Cortesini *et al*[114] reported a decrease in VIP density, but an increase in NO density. While an increase in NO in idiopathic chronic constipation concurs with rat studies in which colonic transit was reduced by NO blockade, it is difficult to correlate the finding of an increase in one (NO), but a decrease in another (VIP), of the proposed inhibitory neurotransmitters of CCM.

To summarise the available evidence concerning inhibition/relaxation in human CCM, histological studies suggested that NO is the predominant inhibitory neurotransmitter, with VIP also present in some NOS-containing inhibitory neurones. Functional studies appear to indicate that NO is the major inhibitory neurotransmitter, with ATP or VIP having a lesser role. There is some evidence to suggest that

defective nitrergic neurotransmission contributes to the 'spastic colon' found in Hirschsprung disease. It is possible that a similar defect in NO/VIP is present in idiopathic chronic constipation, but this needs further clarification.

2.3.3 Interstitial Cells of Cajal:

The interstitial cells of Cajal (ICC) are located within the smooth muscle layers of the gastrointestinal tract. Based on studies in the gastrointestinal smooth muscle of the mouse, ICC have been identified to act as pacemaker cells that generate electrical slow wave activity and thus phasic contractions[49-51]. In addition, it has been proposed that ICC have a role in propagating electrical activity and in mediating neurotransmission. Mutant mice that lack ICC have disordered intestinal motility patterns due to the lack of slow wave generation[50]. As ICC are present in the muscular layers of the human intestine at similar locations to the mouse intestine, there has been recent interest in studying levels of ICC in relation to human gastrointestinal motility disorders.

ICCs have a distinctive ultrastructural morphology, which has enabled identification of these cells in the intermuscular layer and the deep submucosal layer of the mouse intestine[115]. Maeda *et al*[49] used monoclonal antibodies to block postnatal c-*kit* development in mice. C-*kit* is a proto-oncogene encoding for a transmembrane tyrosine kinase receptor. It was demonstrated that c-*kit* blockade led to an absence of slow wave activity in the small intestine. In the same study, it was noted that cells expressing c-*kit* in mouse intestine had a similar distribution to ICC. However, it was reported that it was unclear whether c-*kit*$^+$ cells were identical to ICC.

Huizinga *et al*[50] confirmed the link between c-*kit*, ICC and pacemaker activity. ICCs were identified by selective methylene blue uptake and electron microscopy ultrastructure. W/W^v mice, a strain that have mutations on the c-*kit* gene, were shown to lack ICC in the myenteric plexus. In the W/W^v mice, slow-wave pacemaker activity in the small intestine muscle layers was absent. This concept was extended by Tomsen *et al*[51], who used a patch clamp recording technique to demonstrate that individual ICCs (identified using c-*kit* mRNA expression) generate a spontaneous rhythmic current. *In vivo* correlation of pacemaker activity of intestinal pacemaker activity was reported by Der-Silaphaet *et al*[116], who observed irregular contractions and weak propulsion of intestinal content in W/W^v mice, compared to controls.

The distribution of ICC has been reported in human intestine. Vanderwinden *et al*[52] described ICC distribution, using c-*kit* immunoreactivity, in the colon from 5 'normal' patients (i.e. with no known colonic disease). ICCs were found between the circular muscle and the submucosa and around the deep submucosal plexus. These cells were fusiform in shape with two processes. ICCs with multiple short processes were located around the myenteric ganglia. ICCs were present in fetuses and in neonates, indicating that ICC probably do not differentiate post-natally. He *et al*[53] described ICC in all layers of the sigmoid colon, with a dense network at the myenteric plexus level. Vanderwinden *et al*[52] reported that in the gastric pylorus, ICC appeared bipolar with 1-2 long processes in the muscular layers, whereas ICC with short branching processes were present in the myenteric plexus.

Studies of ICC in relation to several intestinal motility disorders have been reported. In each of these, a lack of pacemaker activity mediated by ICC has been proposed as

contributing to the aetiology of disordered motility. Reduced numbers of ICC have been reported in infantile hypertrophic pyloric stenosis[117], Hirschsprung disease[52], intestinal neuronal dysplasia[118], hypoganglionosis[119], slow-transit constipation[53], adult megacolon[115] and children with anorectal malformations[120].

2.3.4 Electrical basis of contraction:

Some progress has been made in identifying the electrical activity associated with excitation/contraction and inhibition/relaxation in the human colon and in classifying the types of resulting contractions. In particular, there has been a focus on the basis and control of 'migrating motor complexes'. Advances in the understanding of the control mechanisms associated with electrical activity have helped to define the relationship between the circuitry of the enteric nervous system and the resulting physiological colonic contractions. Three types of colonic contractions have been recognised – individual phasic contractions, organised groups of contractions and special propulsive contractions[54]. Individual phasic contractions are the most common type of contractile activity. These occur as isolated units, which can be of short or long duration. Organised groups of contractions are patterns of alternating contractile and relaxed sequences, which for example, have a duration of 7-12 minutes in the dog colon[54, 121]. These contractions often propagate, usually in an anal direction. Contractions that propagate over more than half of the colon have been named colonic 'migrating motor complexes' (MMCs), by Sarna *et al*[122]. MMCs are also a feature of small intestinal motility, where cyclic activity occurs in a more organised fashion[121].

Special propulsive contractions include 'mass movements' and defaecation. Infrequent rapid movement of colonic content was first observed radiologically in humans early in the twentieth century. These contractions were later named 'mass movements'[123]. Rapid propulsion of colonic content in an antegrade direction can occur with or without subsequent defaecation. MMCs are thought to be the underlying mechanism of 'mass movements'[54].

Colonic motor activity is controlled by 3 interacting mechanisms – myogenic, neural and chemical. Myogenic control describes how smooth muscle cells control contractile activity by spontaneous changes in the resting cell membrane potentials. These spontaneous changes, known as 'electrical control activity' (also called 'slow waves') can in turn generate 'electrical response activity' thus causing muscle contraction. Some studies have suggested that electrical control activity may originate from the interstitial cells of Cajal. Electrical control activity exerts influence over short duration contractions. A type of electrical activity known as the 'contractile electrical complex' controls long duration contractions[54].

Three branches of the nervous system influence colonic motility to varying degrees. The central nervous system (CNS) exerts a minimal, largely modulatory, influence. An exception to this is the control of defaecation, during which the CNS co-ordinates external anal sphincter relaxation and the required increase in intra-abdominal pressure. The autonomic nervous system also exerts a mainly modulatory influence, but to a greater degree than the CNS. The parasympathetic branch of the autonomic nervous system has input via the vagal and pelvic nerves, whereas the sympathetic system acts via the splanchnic, hypogastric and lumbar nerves[54, 55].

The enteric nervous system has by far the greatest control of colonic motility, acting via excitatory neurones, inhibitory neurones and interneurones. Some progress has been made in identifying the neurotransmitters involved in the neural mechanisms which control MMCs. Studies in mice appear to indicate that ACh appears to mediate the rapid component of MMCs, while TKs mediate the long duration component. Nitric oxide may maintain inhibition in the periods of quiescence between MMCs[104, 124, 125].

Various chemicals are released by nerve-ending varicosities and endocrine-paracrine cells. These chemicals may influence colonic motility by acting on the ENS, on smooth muscle cells directly, on the CNS, or on the autonomic system. The principal chemicals involved in this mechanism include acetylcholine, tachykinins, noradrenaline, 5-hydroxytryptamine, dopamine, vasoactive intestinal peptide and fatty acid derivatives[54, 55].

2.4 Summary of the Enteric Nervous System:

The enteric nervous system controls gastro-intestinal motility together with lesser inputs from the parasympathetic, sympathetic and central nervous systems. Embryological studies have demonstrated that the ENS originates from the vagal and sacral levels of the neural crest. The ENS has a high degree of complexity and has been extensively studied and mapped in species such as the guinea pig and mouse, but to a much lesser degree in humans. This is partly due to the difficulties with obtaining human gut for experimentation and due to the higher level of sophistication in the human enteric neural circuitry. Intestinal motility occurs via the interaction of primary afferent sensory neurones, interneurones and ascending and descending motor neurones. Rather than functioning as individual reflex pathways, these neurones form a network and a level of modulation and control is established by inhibitory and excitatory interneurones. The interstitial cells of Cajal appear to have a role in generating slow-wave potentials and in modulating enteric neural activity. The principal neurotransmitters involved in mediating excitation/contraction and inhibition/relaxation of intestinal smooth muscle are acetyl choline, the tachykinin peptides, nitric oxide, VIP and ATP. There have been some advances in understanding how activity at the neural level leads to smooth muscle contraction/relaxation and how these contractions are organised and controlled to produce groups of propagating sequences of varying amplitudes and distances and, therefore, how mass movements and defaecation are elicited. However, the intricacy of the ENS means that there are inherent difficulties in studying motility. A range of techniques ranging from intra-cellular recordings, mechanical contractility studies of intestinal muscle strips or sheets, through to investigations of propulsion in intact gut will be required. For ethical reasons, human tissue is rarely available, especially from

healthy subjects and this produces some limitations in the understanding of the ENS and in establishing a background against which motility disorders can de assessed.

2.4 Disorders of colonic motility in children:

2.4.1 Simple constipation:

Constipation in children is very common and such patients form a significant proportion of referrals to paediatric gastroenterology clinics[2]. No cause is identifiable in over 90% of these children. Constipation is defined in relation to the bowel habit seen in healthy infants and children. Clark[126] reported that 99% of healthy term neonates pass the first meconium stool within 24 hours of life and that 100% do so within 48 hours. Weaver and Steiner[127] described that most pre-school children consumed a low fibre diet and passed stool once per day, with 96% opening their bowels with a frequency of between 3 per day and once every second day. It was also found that the frequency of bowel motions decreased between the ages of 1 and 4 years.

More recently, functional constipation has been formally defined by the Rome criteria. In adults, the criteria to diagnose constipation include a bowel frequency of less than 3 times per week, together with straining, hard stools, incomplete evacuation, sensation of blockade and manual manoeuvres to facilitate defaecation on 1 out of every 4 defaecation episodes[128]. In children, functional constipation is defined by the Rome criteria as firm stools 2 or less times per week, with hard pebble-like stools (and the absence of structural, endocrine or metabolic cause)[129]. Soiling as a clinical feature is usually reserved for children over 3 or 4 years of age. Faecal impaction in relation to idiopathic constipation has been defined by Van der Plas *et al*[40] as being either a full rectum on digital rectal examination or rectal enlargement on XR causing a rectopelvic ratio of greater than 0.6.

Functional faecal retention is typified by the infrequent passage of large stools, which often cause anal fissures and rectal bleeding. This leads to stool withholding behaviours, including 'retentive posturing', often mistaken by parents as straining[129]. Stool withholding leads to faecal accumulation and accommodation of the rectum so that the urge to defaecate reduces. Eventually, symptoms such as overflow soiling, poor appetite, abdominal pain and vomiting can occur, while treatments such as laxatives and enemas become increasingly ineffective.

Childhood constipation can be the presenting symptom of various organic and non-organic disorders. Metabolic disorders such as hypothyroidism, diabetes mellitus, coeliac disease and hypercalcaemia, as well as congenital anomalies including ano-rectal malformations and anal stenosis need to be considered and excluded.

2.4.2 Colonic dysganglionoses:

Primary enteric neuronal abnormalities with either increased or decreased neuronal numbers may present clinically with constipation. These colonic dysganglionoses include Hirschsprung disease, intestinal neuronal dysplasia, hypoganglionosis and hyperganglionosis secondary to multiple endocrine neoplasia type 2B. These developmental disorders provide important background to the description of idiopathic colonic dysmotility in children. These conditions are described here in some detail in order to highlight that there is considerable controversy related to diagnostic criteria and that there is a clear gap in knowledge with respect to the histological and physiological characteristics of children with colonic dysmotility.

2.4.2.1 Hirschsprung Disease:

Hirschsprung disease (HD) is a disorder in which there is a total absence of ganglion cells in the myenteric and submucosal plexuses of the distal intestine. Harald Hirschsprung first described congenital megacolon in 1888 and at this stage the dilated colon proximal to the affected portion was assumed to be the pathological segment[130]. It has been recognised subsequently that it is the aganglionic distal colon that is pathological and this affected region remains tonically contracted. Swenson *et al*[131] first reported the absence of the myenteric plexus in affected colonic segments in 1949. The affected segment of colon includes the internal anal sphincter and a continuous portion of rectum and sigmoid colon extending proximally for a variable distance. In approximately 75% of cases, only the rectosigmoid is involved. In the remainder, the affected segment extends more proximally along the colon (17%), and, rarely, the whole colon/distal ileum is involved (8%)[62]. Most cases present in the neonatal period, however, some present later into childhood and even adulthood[132].

HD occurs 1 in 5000 newborns, with males affected 4 times more frequently than females. The frequency in siblings of affected children is approximately 4%[62, 64, 133]. An increased risk is noted with longer segment HD cases, corresponding to autosomal dominant inheritance with incomplete penetrance[61, 64]. HD is associated with several other syndromes, including Down syndrome (trisomy 21), Waardenburg syndrome, Smith-Lemli-Opitz syndrome and congenital central hypoventilation syndrome[62, 64, 133, 134].

2.4.2.1.1 Pathogenesis of Hirschsprung disease:

The pathology underlying HD remains unknown, but is likely to be related to failure of migration of the neural crest cells between the 4th and 12th weeks of embryogenesis. Currently, mutations in at least 8 different genes have been described in association with HD – RET, GDNF, NTN, EDNRB, EDN 3, ECE-1, SOX 10 PHOX2B and SIP 1[62, 64, 134, 135]. The RET tyrosine kinase receptor (chromosome 10q11), which controls the migration of neuroblasts into the fetal gut mesenchyme, shows a mutation in half of familial cases of HD and in approximately 20-30% of sporadic cases. However, only 10% of sporadic short-segment cases have a RET mutation[62, 64]. Glial cell line-derived neurotrophic factor (GDNF) is important in the development of the enteric nervous system. Mice with a mutation in GDNF display renal agenesis and lack an enteric nervous system. In humans, GDNF (and NTN) mutations may be involved in the aetiology of HD, but only in association with a RET mutation. Endothelin-B receptor (EDNRB) mutations occur in 3-7% of HD cases[64].

2.4.2.1.2 Clinical presentation of Hirschsprung Disease:

In the neonatal period, there is a typical triad of presenting symptoms - failure to pass meconium in the first 48 hours of life, bilious vomiting and abdominal distension[136, 137]. Clark[126] reported that 99% of healthy term neonates pass meconium within 24 hours and that 100% did so within 48 hours. Thus, failure to pass meconium within 48 hours is a cardinal symptom of HD. Enterocolitis is the most significant complication of HD and may be the presenting feature. Abdominal distension, fever, severe diarrhoea and rectal bleeding are the symptoms and signs of enterocolitis[138]. The diarrhoea is often caused by *clostridium difficile* toxin and so appropriate antibiotic therapy (for example, vancomycin) is required. If HD presents later in childhood, then

the typical symptoms are chronic constipation, abdominal distension and failure to thrive[131].

The investigation modalities used to diagnose HD are histological assessment of rectal biopsies, radiological contrast enemas and ano-rectal manometry. Rectal biopsies are obtained using a suction device in neonates without anaesthesia[139]. In older children open biopsies are obtained under general anaesthesia. Biopsies are taken at 2, 3 and 4-5 cm above the dentate line and should include mucosa and submucosa to accurately diagnose HD[130, 140]. There is a physiological zone of hypoganglionosis in the distal 1-3 cm of the rectum and so biopsies are required above this level to accurately diagnose HD[131]. The submucosa is examined to confirm an absence of ganglion cells. Biopsies positive for HD also show hypertrophic nerve trunks.

In order to avoid the diagnostic error of sampling the physiological zone of hypoganglionosis, biopsies that appear to lack ganglion cells are stained for the enzyme acetylcholinesterase (the enzyme that breaks down the neurotransmitter acetylcholine). The hypertrophic nerve trunks stain positively for acetylcholinesterase in equivocal cases. Acetylcholinesterase staining has been shown to have a specificity of 100%, as demonstrated by a series of 41 cases in which there were no false positives. However, there is a slightly lower sensitivity of 91%[140].

Radiological contrast enemas can be used to demonstrate the normal or reduced-calibre distal rectum and dilated proximal colon. This may provide a guide to the level of transition to normal ganglionated bowel and so assist in planning surgical

intervention[141]. The use of contrast enemas is somewhat controversial, as a proportion are inconclusive, especially if rectal examinations and rectal washouts have been performed prior to the study[130].

Ano-rectal manometry may be used in equivocal cases of suspected HD. An absent rectoanal inhibitory reflex is typically seen, due to the aganglionosis of the internal anal sphincter. This test has a high sensitivity and specificity (> 90% in some series). However, false positives and negatives can occur[39, 142]. Ano-rectal manometry appears to be most useful for detecting short segments of aganglionosis[143].

2.4.2.1.3 Management of Hirschsprung Disease:

The initial management of HD involves decompression of the colon to allow stool to bypass the obstructed affected segment. This is achieved by regular rectal washouts using normal saline. Surgical correction is required to either bypass or resect the affected aganglionic segment and anastomose normal ganglionic bowel to the internal anal sphincter. The conservative approach to management involves preliminary colostomy formation, then surgical correction at a few months of age[130, 131]. Recently, primary surgical correction in the neonatal period has been successfully employed[144]. In addition, the outcome of trans-anal anastomotic techniques, with either laparoscopic or open pelvic dissection, appears to be comparable to purely abdominal approaches[145, 146].

Three operations have been widely used in the surgical treatment of HD – Swenson's original technique (1948) and the modifications described by Duhamel and by Soave in the 1960's. Swenson's technique involves resection of the affected aganglionic

affected segment and end-to-end anastomosis of normal ganglionic colon to the level 2 cm above the dentate line. In Duhamel's modification, the space behind the rectum is opened and ganglionic colon is 'pulled-through' and anastomosed to the internal anal sphincter. Soave's technique involves an extramucosal dissection of the rectosigmoid and pulling through proximal colon within the established mucosal cuff to the internal anal sphincter[130, 131].

Operative complications of corrective surgery for HD include anastomotic leak (5-7%), pouch impaction and abscess formation[131]. The incidence of longer-term complications is more difficult to define and depends on the method used to acquire follow-up data as well as definitions of terms such as soiling or constipation. Constipation occurs more commonly following the Duhamel technique, whereas soiling is associated with the Soave technique. Despite various reports of favourable long-term outcomes, it appears that on careful follow-up, significant symptoms such as soiling may occur in over half of children and that normal ano-rectal physiology is not restored in the vast majority[144, 147].

2.4.2.2 Chronic intestinal pseudo-obstruction syndrome:

The chronic intestinal pseudo-obstruction syndrome (CIPO) is a group of related disorders in which there is functional bowel obstruction in the absence of mechanical occlusion of the bowel lumen. This syndrome is typified by recurrent episodes of abdominal distension and bilious vomiting in neonates and constipation, bloating and bilious vomiting in older infants. There is a high associated morbidity and mortality. CIPO can be a primary disorder or can occur secondary to infection, electrolyte

abnormalities, connective tissue disorders or drugs. Cases can be either inherited or sporadic[148-150].

Primary or idiopathic CIPO can be subdivided on a histological basis into myopathic and neuropathic cases. In myopathic cases, there is an identifiable defect at the smooth muscle level with necrosis, vacuole formation and connective tissue replacement being typical. In neuropathic cases, the defect is at the enteric neural level and anomalies of the myenteric plexus are seen[150].

Gastro-intestinal conditions associated with CIPO include malrotation, pyloric stenosis and short gut. Associated urological manifestations have been reported, including bladder adynamia or the megacystis-microcolon intestinal hypoperistalsis syndrome[149-151]. The aetiology of CIPO is currently unknown, although some authors have suggested a role for altered pacemaker interstitial cells of Cajal[152].

Clinical investigations used in the diagnosis and assessment of CIPO include histopathological assessment of full-thickness intestinal biopsies, manometry (oesophageal, small intestinal and anorectal, any of which may demonstrate reduced motility) and contrast studies (to exclude mechanical causes of obstruction)[149, 150]. Urological investigation includes ultrasound and micturating cystography[151].

Initial management of cases of CIPO involves correction of associated fluid and electrolyte derangements and antibiotic therapy for gut bacterial overgrowth. Pro-motility agents generally are ineffective. Cisapride (now withdrawn from use in the United Kingdom due to cardiac adverse-effects) has previously been shown to

provide short-term relief in only a minority of patients. Total parental nutrition is required in cases where intestinal failure occurs. Treatment can be necessary for several years and central venous catheter-related complications are common[149, 150]. The role of surgical intervention in cases of CIPO is essentially restricted to the formation of a defunctioning ileostomy. In a series reported by Heneyke *et al*[149], ileostomy formation provided symptomatic relief in 25%, was partially successful in a further 25%, but unsuccessful in 50%. Functional obstruction can occur even in the presence of a normally acting stoma.

2.4.2.3 Intestinal neuronal dysplasia:

The diagnosis 'neuronic dysplasia of the colon' was first used by Meier-Ruge in 1971 to describe histopathological changes seen in the submucous and myenteric plexuses (SMP and MP) of the colon and rectum of children who presented with a primary colonic dysfunction and in whom Hirschsprung disease had been excluded[153]. Neuronic dysplasia of the colon is essentially synonymous with intestinal neuronal dysplasia (IND) and neuronal intestinal dysplasia (NID).

Whilst it is generally agreed that non-Hirschsprung colonic dysmotility in children is a distinct and important clinical entity, the criteria used for the diagnosis of intestinal neuronal dysplasia remain highly controversial. Reports entitled 'Back to the drawing board: IND-B – not a histological entity yet'[27] and 'Intestinal neuronal dysplasia. Why does it only occur in parts of Europe?'[154] highlight the degree of scepticism held by many pathologists and surgeons. Csury and Pena[28] published a review of the literature related to IND in 1995, which underlined that there appears to be a European-bias towards acceptance of the diagnosis. Since the first description of IND

in 1971, attempts have been made to refine and standardise the diagnostic histological criteria, notably in 1981 and 1994[155, 156]. The evolution of these criteria and their potential physiological and clinical correlations will be discussed in this section, in order to examine the usefulness of the term intestinal neuronal dysplasia.

The historical development of the diagnosis of intestinal neuronal dysplasia relates to the use of rectal biopsy as a tool for identifying Hirschsprung disease. Suction rectal biopsies taken at 2, 3 and 4-5 cm above the dentate line are the standard test for Hirschsprung disease. This biopsy technique samples the submucosa, with the absence of ganglion cells and a positive acetylcholinesterase reaction being highly specific and sensitive for diagnosis. However, in children with functional bowel obstruction, but in whom normal ganglion cells were seen on rectal biopsy, Meier-Ruge and Schärli proposed that alternative abnormalities seen in the SMP and MP might be used to classify an alternative disease process which they called 'intestinal neuronal dysplasia'[155-157].

The original histological description of intestinal neuronal dysplasia focussed on the appearance of the SMP and MP ganglia and on the acetylcholinesterase reaction (identical to that used to support the diagnosis of Hirschsprung disease)[155]. The SMP and MP were described as hyperplastic with 'giant ganglia' and there was associated hypoplasia or aplasia of the sympathetic neurones. Based on a relatively small number of cases, it was suggested that localised and disseminated forms of the disease might exist. Localised forms of the disease were noted to present with bowel obstruction and responded to resection of the affected segment. The disseminated form presented with bloody diarrhoea, was unlikely to respond to resection or diversion and was usually

fatal. At this stage it was also noted that the histological changes of intestinal neuronal dysplasia could also occur in the ganglionated segment or the transition zone of Hirschsprung disease colon.

Following these original descriptions, increasing numbers of patients with intestinal neuronal dysplasia were identified and the subclassifications of IND-A and IND-B were made. The histological criteria were revised and reported by Meier-Ruge in a large series of 773 rectal biopsies in 1994[156]. 145 of these patients were diagnosed as having features of isolated intestinal neuronal dysplasia and a further 64 had features of intestinal neuronal dysplasia co-existing with aganglionosis in the distal colon. This study proposed that the most important feature useful to diagnose intestinal neuronal dysplasia was the presence of giant ganglia in the SMP (2 to 3 times the normal size), with > 7 nerve cells per ganglion. Additional features were: a moderate increase in the acetylcholinesterase reaction, 'nerve cell clusters' and nerve cells present inside afferent fibres. These findings were present in 26% of the 774 rectal biopsies evaluated and this group of IND features formed the subgroup IND-B.

IND-A was described as hypoplasia or aplasia of the sympathetic innervation of the SMP. It was seen much less commonly, occurring in only 1% of biopsies assessed[156]. The clinical scenario associated with IND-A was of neonatal enterocolitis - bloody diarrhoea and abdominal distension. However, due to its rarity, IND-A was not reported often and the term has fallen out of use. Subsequent reports of IND generally referred to the features described for IND-B.

It was recognised early on that the histological criteria of IND were also present in a proportion of proximal colonic segments in cases of HD[158]. The incidence of this finding been estimated to be approximately 25 – 45 %[159-162]. It was proposed that proximal segment IND could be a cause of post-operative bowel dysfunction following pull-through surgery in HD patients. Koboyashi *et al*[163] noted that 10 out of 14 patients with continuing bowel symptoms following HD surgery had IND features in the proximal colon. Schulten *et al*[164] reported that patients with IND in the proximal segment had a slight increase in the incidence of constipation, compared to those with normal histology of the proximal segment.

The incidence of IND has been estimated as being between 1 in 4000 and 1 in 60,000 live births[165]. IND has been reported as making up between 5 and 25% of colonic dysganglionoses diagnosed on rectal biopsy[160, 165].

The clinical features of intestinal neuronal dysplasia vary according to the age of the patient. If the presentation is in the neonatal or early infancy period, then the clinical picture is similar to HD with delayed passage of meconium, abdominal distension and vomiting. Children who present later in childhood typically have infrequent bowel actions, abdominal pain, bloating and failure to thrive[166]. It is unclear why children can present at different ages, as the abnormalities of intestinal neuronal dysplasia are thought to be congenital. However, it is possible that this is related to postnatal maturity of the enteric nervous system.

A wide range of gastro-intestinal and extra-gastro-intestinal anomalies have been reported as occurring with IND. These include oesophageal atresia, intestinal atresia,

pyloric stenosis, anal atresia, Down syndrome, megacystis and mental retardation[162, 167].

The treatment advocated for IND varies according to the age of the patient, the length of illness and also whether there is co-existing aganglionosis. Schmittenbecher *et al*[161] recommend that in cases of HD-associated IND, limited resection of the affected proximal segment should be undertaken. However, Koboyashi *et al*[163] suggest that isolated HD-associated IND often resolves with age and so surgery should be avoided if possible. Again, this phenomenon may be related to maturation of the enteric nervous system.

Schärli[166] has recommended conservative therapies for at least 6 months, proceeding to posterior anal sphincteromyotomy in resistant cases. Colonic resection may be undertaken if gross dilatation and failure of propulsion occurs. Diverting colostomy may be required in cases of early onset bowel obstruction or enterocolitis[165].

In a series of adults with IND, surgical intervention was required in 24 out of 36 adults diagnosed with colonic dysganglionosis. Internal anal sphincterotomy was undertaken in 12, sigmoid colectomy in 2 and subtotal colectomy in 10[168].

With the spectrum of therapies advocated, it is difficult to ascertain a reliable prediction of functional outcome with respect to IND. Gillick *et al*[169] reported normalisation of bowel habit in 64% of patients managed on conservative therapies. 36% required sphincterotomy and half of these had a good outcome[169]. Schärli has

reported a 90% satisfactory outcome in patients undergoing sphincterotomy. Colonic resection was successful in all 6 patients in whom this treatment was required[166].

The aetiology of IND is currently unknown. It has been proposed that the neuromuscular junction may be defective, as determined by reduced immunoreactivity in IND colon for neural cell adhesion molecule, gap associated protein-43 and synaptophysin[170]. Miyazaki *et al*[171] used NADPH-diaphorase staining as a marker for neurones containing nitric oxide. They suggest that the reduced NADPH-diaphorase staining seen in HD and allied disorders may be sufficiently sensitive to be useful as an adjunct in diagnosing these conditions on rectal biopsy.

Wheatley *et al*[6] reported reduced levels of substance P in the colonic circular muscle of children with severe slow-transit constipation. It was proposed that this might represent a variant of IND[172]. To address the issue of whether reduced SP staining in slow-transit constipation children might correlate with IND, a further study by Imaji *et al*[173] demonstrated that 8% of these children had both reduced SP staining in the colonic circular muscle and IND-type giant ganglia/increased nerve cell numbers per ganglion. Therefore, it was proposed that patients with the histological characteristics of IND might represent a small subset of children with slow-transit constipation and reduced substance P nerve fibre density.

Familial cases of IND have been studied to assess whether the progress made in defining the genetic basis of Hirschsprung disease might be applicable to IND.

However, it appears that mutations in the RET proto-oncogene, for example, are not associated with IND[174].

Having outlined the background to IND, together with its relationship to HD, its presentation and possible treatment strategies, it is important to consider the controversies with respect to the diagnostic criteria for IND. The consensus for the diagnostic criteria described by Meier-Ruge *et al*[156] in 1994 has been questioned by several authors. Indeed some have questioned the existence of IND as a disease entity. Koletzko and Meier-Ruge[175] reported a multicentre prospective study, which assessed the degree of interobserver error with respect to the histological diagnosis of HD and IND on 108 rectal biopsies. Three pathologists independently assessed the same biopsies. There was agreement on the diagnosis of aganglionosis, but wide variability with respect to IND classification. Lumb *et al*[27] also concluded that the histological criteria for IND were not sufficiently specific, on the basis that giant ganglia form 10% of the spectrum of ganglia seen in adult colon resected for carcinoma and that ganglia with > 7 nerve cells per ganglion are found in > 50% colonic specimens taken from non-constipated adults. IND-type findings have also been described in other paediatric colonic specimens, in association with ano-rectal malformations, cow's milk intolerance and neurofibromatosis[176].

A further criticism of IND is that no physiological defects (*in vitro* or *in vivo*) have been described which might correlate with the histological changes seen. Koletzko *et al*[177] investigated colonic transit time and ano-rectal manometry in children with IND. They suggest that there was a significant reduction in the recto-anal inhibitory reflex (normally seen in response to rectal distension) in IND patients, but that other

manometric and transit findings were similar in IND and non-IND constipated children.

The problem remains as to whether the changes of giant ganglia, with an increased number of nerve cells, are useful in characterising children with non-HD colonic dysmotility. It seems that the term 'intestinal neuronal dysplasia' has some use in that it refers to a group of children, who do not have Hirschsprung disease, but who do have a functional bowel disorder, which is likely to be due to abnormalities within the enteric nervous system. However, it seems likely the IND criteria identify only a small subgroup of children with functional bowel obstruction. The histological criteria for IND proposed in 1994 do not have the required specificity to allow classification of this group of children, nor do they allow decisions regarding specific operative or non-operative treatments strategies to be made. Clearly, there is a lack of knowledge relating to the histological and physiological abnormalities of children with colonic dysmotility in whom HD has been excluded.

2.4.2.4 Hypoganglionosis:

Hypoganglionosis is the third commonest colonic dysganglionosis, which like Hirschsprung disease and intestinal neuronal dysplasia, presents as functional colonic obstruction either in the neonatal period, or during infancy[178]. Although hypoganglionosis is considered to be allied to Hirschsprung disease, it differs from aganglionosis in certain important aspects, both in terms of clinical presentation and pathological basis. Hypoganglionosis refers to an abnormality of the myenteric plexus in which there is a decrease in the number of nerve cells, an increased interganglia distance and a reduction in the myenteric plexus surface area. It was first described by

Bentley in 1964 in relation to megacolon in childhood[179]. The incidence of hypoganglionosis has been estimated as approximately 1 in 100,000 and it has been demonstrated in approximately 5% of a large series of rectal biopsies[178, 180].

Like intestinal neuronal dysplasia, hypoganglionosis has been reported as occurring in localised and disseminated forms and as occurring in association with Hirschsprung disease[178]. Again, in common with intestinal neuronal dysplasia, hypoganglionosis is a controversial disease entity. Smith[181] has estimated the normal neuronal density in child colon to be 7.7 per mm, which essentially did not vary with age until late into adulthood. However, it was noted that this value varied hugely in other studies, as Schuffler *et al*[182, 183] had reported a value 20 times lower than 7.7 per mm and Meier-Ruge *et al*[184] had reported a value 10 times higher. Therefore, pathological diagnostic criteria to define reduced levels of ganglia are not well characterised.

Schärli and Meier-Ruge[178, 185] reported the criteria most commonly referred to, in a series of 353 rectal biopsies, of which 15 were described as having the features of hypoganglionosis. These criteria consist of an increase in the interganglia distance (by 2 times), a decrease in the number of nerve cells in the myenteric plexus, and a decrease in the surface area of each myenteric plexus (by half). An increased interganglia distance and a decreased mean surface area of the myenteric plexus were also features of Hirschsprung-associated hypoganglionosis. However, these parameters were not so marked as in isolated hypoganglionosis.

Several alternative methods for the pathological evaluation of hypoganglionosis have been described. Kubota *et al*[180] described measuring the proportion of the myenteric plexus, which stained positively using fluorescence immunohistochemistry. This ratio was found to be significantly lower (when compared to normal neonatal colon) in 6 cases of 'Hirschsprung-allied disorder'. Watanabe *et al*[186] emphasised the appearance of the hypoplastic nerve fibres seen in hypoganglionosis, as compared with the hyperplastic thick nerve fibres seen in Hirschsprung disease. Ariel *et al*[186] reported a case of hypoganglionosis of the myenteric plexus, associated with normal appearances of the submucous plexus. These authors, together with Schärli *et al*, argue that hypoganglionosis is a disorder affecting the myenteric plexus, and therefore, it should only be diagnosed on full thickness colonic biopsies and not on rectal suction biopsy alone.

Hypoganglionosis presents with typical symptoms of functional obstruction – constipation, soiling, abdominal distension and vomiting. Kobayashi *et al*[187] suggested subgroups of hypoganglionosis – type A and type B. Type A referred to a shorter segment of hypoganglionosis, with a good prognosis. Type B represented a longer segment of affected colon and small bowel, which presents as a severe congenital enterocolitis and had a very poor outcome. Schärli[178] classified hypoganglionosis as occurring in localised, disseminated and Hirschsprung-associated forms. Localised forms behaved similiarly to long segment Hirschsprung disease and responded to Soave-type resection. Disseminated forms did not respond to resection, and instead patients subsequently became dependent on total parenteral nutrition.

There are no sufficiently specific abnormalities on clinical investigations, such as Xrays, colonic transit studies and ano-rectal manometry, to allow accurate diagnosis of hypoganglionosis[188]. Rectal suction biopsy is essential to exclude Hirschsprung disease. However, as outlined above, most authors suggest that full thickness rectal or colonic biopsies are required for the diagnosis, as the pathological changes occur in the myenteric plexus and not in the submucous plexus.

Although there is not a consensus on the optimal treatment for hypoganglionosis, surgical rather than conservative management is generally advocated for all types except the disseminated form. Schärli[178] noted that Hirschsprung-associated hypoganglionosis (usually involving a short segment) should be resected at the time of definitive surgery for the aganglionic segment. The same author reported a good outcome following Soave procedure for localised, isolated cases. Ariel *et al*[189] reported a case of hypoganglionosis in a 16 year old girl, who responded well to a Duhamel-type procedure. Meier-Ruge *et al*[185] suggest that hemicolectomy produces a favourable outcome. Disseminated forms are not amenable to surgery and are likely to require long periods of parenteral feeding. It should be noted that there are no large series reporting treatment outcome for hypoganglionosis. All of the reports outlined here are of small series of less than 10 patients of each type of hypoganglionosis.

The aetiology of hypoganglionosis is unknown, although some recent reports have shed some light on possible causative mechanisms. Schärli[178] has suggested that the disorder may be a congenital hypoplasia of the myenteric plexus, or may be secondary to abnormal expression of trophic factors. Rolle *et al*[190] and Yamataka *et al*[191] have reported abnormalities in the pacemaker interstitial cells of Cajal, as determined by c-

kit expression in cases of hypoganglionosis. Inuoue *et al*[192] report that, unlike in a proportion of Hirschsprung disease patients, there were no mutations of the RET or GDNF genes contributing to 5 cases of hypoganglionosis.

In summary, hypoganglionosis can be seen to be a somewhat controversial disease entity, similarly to IND. The normal density of neurones in the human colon is disputed, making it difficult to define a reduced density or hypoganglionosis. This further emphasises the need for research into potential enteric nervous system abnormalities in children with colonic dysmotility.

2.4.2.5 Multiple endocrine neoplasia:

Multiple endocrine neoplasia type 2B (MEN 2B) is considered to be a form of hyperganglionosis in which transmural ganglioneuromas occur in the gut. The disorder has an incidence of 1 per 30,000[65]. It is an autosomal dominantly inherited disease. In MEN 2B there is a mutation in the tyrosine kinase domain of the RET proto-oncogene on chromosome 10p11[193]. The RET proto-oncogene codes for a tyrosine kinase receptor which is expressed in cells derived from the neural crest. The clinical features are of multiple mucosal ganglioneuromas, such as full lips, marfanoid features, large corneal nerves, phaeochromocytomas and medullary thyroid carcinomas. Gastro-intestinal manifestations are of constipation and diarrhoea, mega-colon or obstruction. Ganglioneuromatosis affects neurones, glial cells and nerve fibres, which appear thickened[65]. As the risk of medullary thyroid carcinoma is 100%, DNA testing is advised for all those at risk, including those who have transmural ganglioneuromatosis seen histologically on colonic biopsies performed at the time surgical correction of Hirschsprung disease[194, 195].

2.5 Clinical investigation of constipation in children:

The clinical investigations commonly used to assess children with chronic constipation include rectal suction biopsy, gastro-intestinal transit studies and ano-rectal manometry. Rectal suction biopsy is the gold standard investigation for the diagnosis of Hirschprung disease and is described in section 2.4.2.

2.5.1 Transit Studies:

Transit studies are commonly employed to provide an assessment of gastro-intestinal motility in children. Slow colonic-transit is a well-recognised condition in adults that affects predominantly females[20, 21]. However, there are two important issues relating to gastro-intestinal transit in children. Firstly, what is the normal range of colonic transit in healthy children and, therefore, how should slow-transit in children be defined? Secondly, what is the optimal method of measuring colonic transit? To address the latter question, a review of the different types of transit study has been undertaken, to identify the advantages and disadvantages of each. By combining the results of studies of transit in healthy children, an estimate of normal transit in children can be determined and a definition of slow-transit in children is proposed.

As is the case with many clinical investigations, there is no universal agreement on the optimal technique to derive normal ranges in healthy adults and children and so criteria for disease states are also controversial. It is problematic for ethical reasons to study healthy volunteers using techniques that employ radiation. Thus, there are difficulties in validating new techniques to study colonic transit.

Studies of bowel transit have increased in sophistication from early methods, which measured ingestion and evacuation of markers or dyes (such as carmine)[15, 16], to X-ray studies of ingested radio-opaque markers[12, 196] and gamma camera imaging of ingested radioisotopes[197]. This has allowed estimates of the normal ranges of gastric emptying, small bowel emptying and segmental colonic transit to be defined in healthy children and adults[196]. Transit studies have been applied to the investigation of colonic motility disorders, with the aim of directing medical and surgical therapies more accurately[17, 198]. Subtypes of chronic constipation have been proposed – normal transit, faecal retention/distal delay and slow-transit constipation, which each have different treatment strategies[6, 18].

The simplest studies of bowel transit involved the ingestion of dyes such as carmine, which stain the bowel contents, or the ingestion of beads or seeds, which are retained in the stool. Faeces subsequently evacuated are monitored for the presence of the beads or seeds, or for changes in colour, in order to obtain an estimate of mouth to anus transit time. Dimson described the use of carmine to diagnose constipation in children, stating that the stool passed on day 4 should be free of carmine dye in normal children[15]. It was reported that this was a useful and reliable method of confirming clinical symptoms and signs. This carmine dye technique was used by Weaver *et al*[127] to further define normal mouth to anus transit times in 35 healthy children. Overall, mean transit time was 33 hours. This was slightly faster in children aged less than 3 years (30 ± 18 hours), and slightly slower in those aged 3-4 years (36 ± 13 hours).

2.5.1.1 Radio-opaque marker studies:

Hinton *et al*[14] first described the use of radio-opaque markers to measure mouth to anus transit and this method has subsequently been applied to healthy children[16]. At this stage, radio-opaque marker studies involved the ingestion of plastic pellets and determining the proportion of markers in evacuated stool by X-ray. Burkitt *et al*[16] reported that the mean mouth to anus transit time in teenage boarding school pupils was 76.2 hours (range 35-120 hours). The method described by Hinton *et al* was also used by Cucchiara *et al*[16] to investigate healthy children, to obtain 'control' data to compare with constipated children (with or without faecal soiling). Total gastro-intestinal transit time was reported as 25.6 ± 3.7 hours (range 19-33 hours) in healthy children and was 60.7 ± 18.5 hours in constipated children.

Radio-opaque markers used in conjunction with X-rays provide further information on bowel transit. Colonic segments according to bony landmarks and gaseous colonic outlines have been delineated. Formulae were derived to standardise interpretation of transit with respect to the quantity of markers used and the timing of ingestion[196]. It has been reported that the accuracy of the estimate of bowel transit can be increased by using different radio-opaque markers on different days of the study and by taking multiple X-rays[12, 196]. In an attempt to overcome the risks of radiation exposure, simplified methods have been proposed, based on the use of a single X-ray[12, 198].

Arhan *et al*[196] reported segmental transit times, measured using radio-opaque markers, in healthy adults and children. Bony landmarks and colonic gaseous outlines were used to define 3 colonic regions: right colon, left colon and the rectum. Overall,

it was reported that the total mean transit time was not significantly different in adults and children (38.9 and 28.8 hours respectively). However, transit in the right and left colon was significantly faster in children, compared to adults (13.8 and 14.1 hours in adults; 7.7 and 8.7 hours in children).

It was subsequently suggested that the method of Arhan *et al* employed a radiation dose that was unacceptably high, as daily X-rays were used. Therefore, Metcalf *et al*[12] compared a simplified measurement involving the daily ingestion of 3 different types of radio-opaque markers and a single X-ray on day 4 with a multiple X-ray technique. This was reported to reduce the radiation dose to 1/16 of that of multiple X-rays and to produce comparable data concerning transit. Segmental transit in 24 healthy adults was estimated at 11.3 ± 1.1 hour in the right colon, 11.4 ± 1.4 hour in the left colon and 12.4 ± 1.1 hour in the recto-sigmoid. The total colonic transit time was calculated as 39.9 hours. However, it was acknowledged that this method would underestimate transit in patients with delayed transit greater than 72 hours. A further X-ray on day 7 was recommended.

Several authors have used radio-opaque markers with abdominal X-rays, in order to measure segmental and total colonic transit in healthy children. These findings have been compared with transit studies in constipated children[13, 199-201]. Bautista Casanovas *et al*[200] employed the method of Metcalf *et al*[12] in healthy and constipated children, although the likely underestimation of transit in patients with delayed transit was acknowledged (as it was by Metcalf *et al*). The radio-opaque markers used were not of different shapes, as it was noted that the information obtained from the different shaped markers was not utilised in the formulae to derive

transit. Segmental colonic transit times reported were: 10.8 ± 3.5 hours in the right colon, 12.2 ± 2.7 hours in the left colon, 14.8 ± 2.2 hours in the recto-sigmoid and a total colonic transit time of 37.8 ± 6.2 hours. Therefore, the upper limit of normal transit was defined as 50.2 hours (mean + 2 standard deviations).

Segmental colonic transit in adolescents (12-18 years) has been studied, again using the method of Metcalf *et al.* Median total colonic transit time was reported as 30.2 ± 13.2 hours (range 10.8 to 50.4 hours). Median transit through the right colon was 6.7 hours, 8.0 hours through the left colon and 15.6 hours through the recto-sigmoid region. Total colonic transit, right colonic transit and left colon transit were significantly delayed in adolescents with constipation[201].

Gastro-intestinal pH and transit have been correlated using a radiotransmitting pH capsule, the location of which was identified using fluoroscopy. A drop in pH from 7.4 to 5.9 was used as supplementary indication that the capsule had passed from the terminal ileum into the caecum. This report estimated the median colonic transit time in 12 children with a median age of 12 years to be 17.5 hours[199].

Delayed colonic transit longer than 100 hours (7.9 days) was proposed by Benninga *et al*[13] in an attempt to define paediatric slow-transit constipation as a disease entity. This definition of 100 hours was derived from the upper limit (mean + 2 standard deviations) reported by Corriazzi *et al*[11] in a study of children with constipation. However, as this is selecting the upper limit of transit in children already identified to suffer from constipation, this is likely to be too high to be a useful definition of slow-transit constipation.

Colonic transit has been investigated in other disorders affecting colonic motility, again using radio-opaque markers. Mean colonic transit times were reported as being significantly slower in children with myelomeningocele (103.2 ± 49 hours) compared to healthy children (23.3 ± 13 hours)[202]. An alternative method of interpreting radio-opaque marker ingestion with a single X-ray was used to measure colonic transit in healthy children and in children with previous anorectal malformations[198]. Radio-opaque markers were ingested every day for 7 days, at which point it was assumed that a 'steady state' between marker input and output had occurred. Healthy children had a total colonic transit of 1.3 days (range 0.4 to 3.4 days). Children with previous anorectal malformations (both high and low) had significantly delayed colonic transit.

2.5.1.2 Scintigraphy:

Scintigraphy involves the detection of emission of gamma radiation by an ingested radionuclide-labelled substance using a gamma camera and, thus, transit through the whole bowel can be traced. This technique was established first in a study of colonic transit by employing non-physiological conditions. Small bowel intubation was used to instil boluses of radiolabelled tracer directly into the caecum and so estimate subsequent distribution through the colon[197]. Delivery of a bolus of tracer into the caecum has also been achieved using a gelatin capsule with a pH sensitive coating[203].

Subsequent reports have demonstrated that radiolabelled tracer taken orally can be monitored through the whole bowel[204]. Multiple images are possible, without increasing radiation exposure, at short intervals for the first 9 hours and then less frequently up to 48 or 72 hours. Gastric and small bowel transit can been assessed and

further information on segmental colonic transit can be measured. Oro-caecal transit can vary from 2-10 hours, thus potentially influencing assessments of colonic transit[19].

In order to facilitate comparison of patient groups, several authors have used the geometric centre (GC) of radioactivity of the ingested tracer, to provide a single index of transit for each time at which images are acquired. The GC represents the position of the median of radioactivity (i.e. the central area of radioactivity), with 50% of radioactivity being on either side of this point[197, 204 18]. To calculate the GC, 'regions of interest' (ROI) are assigned to the colon. For example, Notghi *et al*[19] used 5 ROI, 1 being the proximal colon through to 5 being the evacuated faeces. Determining the fraction of activity in each ROI, multiplying this fraction by the ROI number, then adding each of these, then calculates the GC. In a study of healthy adult volunteers undergoing scintigraphy, mean arrival time of tracer in the caecum was 4.7 hours, in the transverse colon was 17.4 hours, at the splenic flexure was 25.9 hours, in the rectum was 35.8 hours and in the faeces was 44.9 hours[19]. Several authors have reported that analyses that use GC only are inadequate and that visual assessment on acquired images is important to interpret transit[6, 18].

Scintigraphy has been applied to the investigation of dysmotility in adults and children. This has led to subgroups being proposed, based on types and primary colonic location of transit delay. Notghi *et al*[18] identified 5 types of colonic transit in adults with irritable bowel syndrome and chronic constipation – rapid transit, intermediate transit, generalised delay, right-sided delay and left-sided delay.

This technique has a number of advantages. A more accurate estimate of colonic transit (rather than total gastro-intestinal transit) is possible for the same radiation dose as radio-opaque marker studies, as the timing of the arrival of tracer in the caecum can be measured. Gastric and small bowel emptying can be assessed during the same study. A small proportion of patients with chronic constipation have gastric, small bowel and colonic motility defects. Some authors report the identification of previously undiagnosed pan-gastro-intestinal motility defects using scintigraphy[6, 17]. However, in a series in which oro-caecal transit was measured using a hydrogen breath test (in response to lactulose), no such cases were recognised[13]. Disadvantages of scintigraphy are that more extensive monitoring of patients is required, with an associated increased cost.

2.5.1.3 Radio-opaque markers compared to scintigraphy:

Radio-opaque marker and scintigraphic studies have been compared to determine whether transit is estimated differently by the 2 techniques. In adult volunteers who ingested radiolabelled ^{111}Indium pellets and opaque markers simultaneously, mean total colonic transit was estimated as 35.7 hours and 25.6 hours respectively[203]. It was suggested that this might relate to particle size – up to 6 mm in opaque markers and 0.5-1.8 mm in the radiolabelled pellets. However, in a similar study of ^{111}In-labelled food and opaque markers in healthy volunteers and adults with chronic constipation, no differences in transit estimation was found at any stage[204].

It can be concluded that in contrast to radio-opaque marker studies, scintigraphy provides information on gastric and small bowel transit and so estimates colonic transit more accurately, as well as providing additional information on segmental

colonic transit. Therefore, scintigraphy can be considered to be the 'gold standard' transit study for identifying locations of delayed transit. It appears that radio-opaque marker studies are useful for simple evaluation of chronic constipation. Scintigraphy allows subgroups of patients with constipation to be identified and thus treatments to be better directed. In patients in whom partial colonic resection is being considered, scintigraphy would be required[204].

2.5.1.4 Meta-analysis of transit studies in healthy children:

A Medline search using the terms 'gastro-intestinal', 'colonic', 'transit' and 'children' was undertaken to identify all studies post-1966, which included transit studies in 'controls' or healthy children. 9 studies were found, 1 using carmine dye, and 8 using radio-opaque markers[10, 15, 16, 127, 196, 198-202]. Studies that included a range of values or a mean/median with standard deviations allowed estimation of the upper limit of normal. The upper limit of normal was defined either as the highest value in the range quoted, or as the mean/median plus 2 standard deviations.

In these 9 studies, mean colonic or gastro-intestinal transit was measured in a total of 195 children, producing an overall mean of 29.9 hours. The upper limit of normal could be calculated from studies containing a total of 195 children, producing an overall mean of 57.7 hours (Table 1). This calculation provides a basis for defining normal and delayed colonic transit in children.

	Study	No	Age (yrs)	TGITT/ CTT	Right Colon (hrs)	Left Colon (hrs)	Rectum (hrs)	Mean Total Transit (hrs)	ULN
Dimson, 1970	Carmine	65	3-13	TGITT	N/A	N/A	N/A	N/A	<120
Burkitt, 1972	ROM	9	Teen age	TGITT	N/A	N/A	N/A	76.1	35-120
Arhan, 1981	ROM	23	<15	CTT	18 (ULN)	20 (ULN)	34 (ULN)	29	62
Weaver, 1984	Carmine	35 18 17	1-4 <3 3-4	TGITT	N/A	N/A	N/A	33 30 36	N/A 66 62
Cucchiara, 1984	ROM	46	8.1	TGITT	N/A	N/A	N/A	25.0	32.4
Fallingborg, 1990	ROM	12	12	CTT	N/A	N/A	N/A	17.5	(54.7)
Bautista 1991	ROM	10	6-14	CTT	10.8	12.2	14.8	37.8	50.2
Pigeon, 1997	ROM	22		CTT	N/A	N/A	N/A	23.3	49.3
Rintala, 1997	ROM	25	8	CTT	N/A	N/A	N/A	31.2	(81.6)
Zaslavsky, 1998	ROM	13	12-18	CTT	6.7	8.0	15.6	20.2	56.6

Table 1: Results of 9 studies of gastro-intestinal transit in healthy children. Two types of study have been reported – using carmine dye or using radio-opaque markers

(ROM). Studies reported either total gastro-intestinal transit time (TGITT) or colonic transit time (CTT) (both in hours). Transit times (in hours) through the right colon, the left colon and the recto-sigmoid are shown where these were reported. The mean total transit time (in hours) and upper limit of normal (ULN) is also shown. The upper limit of normal was either reported directly or calculated as the mean plus 2 standard deviations.

2.5.2 Ano-rectal manometry:

Faecal continence is achieved by a combination of the action of the internal and external anal sphincters, the pelvic floor and the ano-rectal angle. The internal anal sphincter is responsible for involuntary continence and is controlled by the autonomic nervous system. The external anal sphincter is innervated by the pudendal nerve (sacral segments S2-S4). It is composed of skeletal muscle and is important in the voluntary control of defaecation. Defaecation is achieved by co-ordination of rectal contractions in response to distension with relaxation of the anal sphincters at a socially acceptable time[205].

The measurement of the pressure characteristics and motility patterns in the ano-rectum of children is used as an adjunct to the diagnosis of HD and to assess symptomatic children who have previously undergone surgery for ano-rectal malformations and HD[2, 41, 206]. Ano-rectal manometry does have some applications in the assessment and evaluation of the treatment of children with 'idiopathic' chronic constipation and/or faecal incontinence. However, these indications for its use are somewhat controversial[40, 207].

A manometry catheter is inserted into the rectum and short duration studies are undertaken. Due to the inherent invasiveness of the technique, the importance of gaining the full co-operation of the child and his/her parent to avoid meaningless studies with excessive artefact has been emphasised. It may be necessary to empty the rectum using enemas prior to studying older children[41].

A water-perfused catheter with at least 2 recording side-holes and a proximal inflatable balloon are most commonly used. This allows measurement of anal canal resting/squeeze pressures, anal canal length, rectal sensations, the recto-anal inhibitory reflex and defaecation dynamics. Anal resting and maximal squeeze pressures are useful to assess continence in patients who have undergone correction of ano-rectal malformations or who have spina bifida. In healthy children, the maximal anal squeeze pressure is approximately 70 cm H_20 - this is similar in males and females[208]. The recto-anal inhibitory reflex (RAIR, also known as the rectosphincteric reflex) is assessed by inflation of the rectal balloon and is positive (i.e. normal) if the resting tone of the internal anal sphincter decreases by more than 5 mmHg[39, 41]. The technique can be applied even to premature neonates, as they have been demonstrated to have anorectal pressure characteristics and an RAIR similar to that seen in older infants[209].

Absence of the RAIR is a consistent feature of Hirschsprung disease, with a sensitivity and specificity variously reported as being greater than 90%. However, the gold standard for the diagnosis of Hirschsprung disease remains histological analysis of rectal biopsies, as false positives and negatives can occur with ano-rectal manometry[142, 210]. Ano-rectal manometry appears to be most useful for detecting short segments of aganglionosis[143].

Ano-rectal manometry has been used in the assessment of children with chronic constipation, with mixed results. An increase in the maximum anal squeeze pressure has been found in approximately 50% of cases in a series of children with chronic constipation, whereas reduced rectal sensations (in response to distension) have been

described in two thirds of children with constipation[208]. Sutphen *et al*[211] reported that there is a paradoxical increase in external anal sphincter pressure during attempted defaecation in children with chronic constipation and soiling, but that overall, there was a lack of manometric correlation with symptoms in this group. Ability to expel a rectal balloon (100 mls) has been reported in association with constipation and soiling in children[212]. Although symptomatic children in this study were more likely to recover if they were able to voluntarily expel the rectal balloon, this feature did not allow prediction of resolution of symptoms[212]. Van der Plas *et al*[40] reported that no significant ano-rectal manometric abnormalities could be detected in children with clinical functional faecal retention. Ano-rectal manometry has also been studied as a biofeedback aid to conventional laxative treatment in children with chronic constipation. Although a significant increase in the maximal achievable squeeze pressure was recorded after two sessions of manometry/biofeedback, there was no improvement in outcome compared to laxatives alone[207].

2.5.3 Colonic manometry:

Colonic manometry is the measurement of *in vivo* pressure characteristics within the lumen of the colon. A catheter with multiple sideholes is inserted into the colon. Pressure transducers in continuity with the sideholes continuously record pressure changes within the colon. Colonic manometry is used as a routine investigation of colonic dysmotility in only a few centres and is mainly employed as a research tool. Manometric techniques are also used to investigate oesophageal and upper small intestinal motility dysfunction[41].

There are two types of colonic manometry systems – water-perfused and solid-state[213, 214]. Water-perfused catheters are used more commonly. Catheters with up to 16 sideholes have been used to carry out multipoint manometry studies[1]. They are cheaper and more robust than solid-state catheters. Solid-state systems, although more fragile and expensive, have the advantage of allowing ambulatory studies to be conducted and so more physiological assessments of colonic motility can be undertaken[214]. No studies, however, have been reported in which solid-state catheters have been used to record simultaneously from the whole colon.

Two methods of colonic manometry catheter insertion have been described, endoscopic placement and nasocolonic catheter advancement. Placement of a catheter over an endoscopically placed guidewire has been undertaken in both adults and children. This method is relatively quick and reliable. However, colonoscopy requires sedation in children and adults and this may suppress colonic motility[215]. In the majority of reports in which endoscopy has been used, the catheter is placed only as far as the transverse colon and, thus, the studies are limited to the distal half of the colon[43, 44, 213]. In addition, the presence of a catheter passing externally through the anus is likely to interfere with colonic motility and so produce less physiological recordings, even when solid-state transducers are used[214].

Nasocolonic catheter placement has been described in adult colonic manometry studies[1, 216, 217]. A long catheter (typically 3 metres) is progressively advanced under fluoroscopic control through the nasum, the oesophagus, the stomach and the small bowel into the colon. The advantages of this technique are that when the catheter is in place, simultaneous recordings can be performed from the whole colon.

As the catheter does not pass through the anus, prolonged recordings are possible. The disadvantages of nasocolonic placement are that intubation takes up 72 hours in total and repeated nasal advancements of the catheter are required. This means that the technique would not be well tolerated by children.

Colonic manometry can be carried out with the colon in the unprepared state or in the prepared empty state by prior administration of laxatives[1, 216]. Studies in the unprepared state would be expected to provide more physiological estimates of colonic motility. However, catheter placement may not be possible in some patients with severe colonic faecal loading without bowel cleansing. In a cross-over study of colonic manometry in the prepared and unprepared state in the same patients, it was reported that overall motor activity was the same, but that there was a difference in the frequency of high-amplitude contractions recorded[216].

Several patterns of motor activity have been recognised using colonic manometry. Pressure 'events' within colonic regions are defined in terms of whether they are part of a sequence that propagates within the colon. Propagating pressure sequences occur in both antegrade and retrograde directions and are thought to be associated with movement of content[1, 218, 219]. However, it has been demonstrated in a study in which manometry was combined with scintigraphy, that not all propagating sequences result in net movement of colonic content[42].

Propagating sequences (PS) can be sub-classified by their amplitude, i.e. the difference in the peak and trough of the pressure event. The importance of this is in relation to high-amplitude propagating sequences (HAPS). These pressure events are

often also termed high-amplitude propagating contractions (HAPC). However, in this thesis, the term HAPS will be used for clarity. As it is unknown if all high-amplitude propagating events are associated with colonic contraction, it is more appropriate to use the term sequence.

HAPS are thought to be the manometric equivalent of radiologically observed 'mass movements'. However, there is considerable disparity in the criteria for a PS being defined as high-amplitude. Indeed, different definitions have been reported in different studies from the same centres. For example, Bassotti *et al*[218, 219] relied on visual identification of HAPS and use the definition of > 100 mmHg. However, in a later series, a definition of < 50 mmHg is applied to differentiate low-amplitude PS[220]. Di Lorenzo *et al* have used various definitions of > 60 mmHg, > 80 mmHg and > 100 mmHg to document HAPS[43, 44, 221]. Bampton *et al*[1] define HAPS in healthy adults as > 116 mmHg, which represents the mean plus 2 standard deviations of colonic pressure at the mid-point of the colon in their studies on healthy adults.

The reported frequency of HAPS is between 4-10 per 24 hours[1, 218, 219]. Antegrade PS occur with a frequency of 50-60 per 24 hours and retrograde PS occur approximately 20 per 24 hours[1, 220]. The daily frequency of HAPS appears to decrease with increasing age through childhood[221]. The mean amplitude of antegrade PS is 20-50 mmHg, of retrograde is approximately 30 mmg and of HAPS is 110-160 mmHg[1, 218, 220]. Defaecation is usually preceded by the onset of colonic HAPS, which may begin up to 1 hour before the passage of stool[217, 221].

Non-propagating motor activity is measured using area-under-the-curve analysis. This provides an adjunct in the interpretation of motor activity associated with activities such as sleep, waking and eating. Studies in healthy adults have demonstrated that there is an increase in the frequency of HAPS and an increase in non-propagating motor activity in response to eating a meal[1, 219]. This 'gastrocolonic response' occurs by a neurally mediated reflex, which is initiated by mechanoreceptors and chemoreceptors in the gastroduodenal wall to stimulate the proximal and distal colon[46]. Meals with a high fat content stimulate motor activity to a greater degree than those that are predominantly carbohydrate[222].

Sleep induces a decrease in PS/HAPS frequency and a reduction in non-propagating activity[1, 46, 219, 220, 223]. The response to waking is characterised by an increase in PS/HAPS frequency and non-propagating activity[1, 219]. The results of these studies on eating, sleep and waking underline the importance of carrying out prolonged manometric studies to fully evaluate colonic motility.

The characterisation of normal values of propagating and non-propagating colonic activity has allowed motility disorders in adults and children to be studied and the motor patterns associated with them to be determined. In adults with idiopathic chronic constipation, HAPS frequency is significantly reduced. Up to one third of patients studied displayed no HAPS in 24 hours[213, 224, 225]. In a proportion of patients with no HAPS, there appears to be an intact colonic motor mechanism, as HAPS can be induced pharmacologically[226]. These patients also lack the increase in motility after meal ingestion seen in healthy adults[213, 224]. Correlation with

symptomatology has been described in a study combining visualisation of the movement of colonic content on scintigraphy with manometry[213]. Constipated adults who demonstrated an increase in colonic motor activity after meal ingestion tended to suffer from chronic abdominal pain and the passage of hard stools, whereas those with no increase in activity had intermittent abdominal pain with nausea and vomiting.

Colonic manometry has also been performed in children with colonic motility disorders[43, 44, 227, 228]. It was demonstrated that children with intractable constipation could be differentiated manometrically into those with functional faecal retention and those with either a neuropathic or myopathic cause for their symptoms[43]. Children with neuropathy or myopathy showed no increase in non-propagating activity or increase in HAPS frequency following ingestion of a meal. Conversely, those with functional faecal retention actually displayed a higher frequency of HAPS than would be expected in adults. Similar findings were determined in children with chronic intestinal pseudoobstruction syndrome, in that patients with a demonstrable myopathy lacked HAPS and lacked a response to meal ingestion[44]. An association with lower urinary tract dysfunction has also been described[229]. Further studies have reported on the manometric characteristics of the colon following corrective surgery for Hirschsprung disease and anorectal malformations[227, 228].

The colon can also be pharmacologically stimulated during colonic manometry studies, particularly in cases where no significant motor activity is detected. This is usually performed by direct instillation of bisacodyl into the lumen of the colon. Bisacodyl is a stimulant laxative that acts directly on colonic sensory nerves, via a

prostaglandin-mediated mechanism, to cause an increase in peristaltic activity[226, 230]. In addition, bisacodyl inhibits water absorption from the colon and so enhances the laxative effect. Typically, defaecation is stimulated in a normal subject 6 hours following oral administration, 15-60 minutes following rectal administration and in < 10 minutes following intraluminal instillation. Bisacodyl-induced HAPS have similar properties to spontaneous HAPS[230]. It was suggested that this method could be used to distinguish patients with a truly 'inert' colon, who may require surgery[226, 230].

The use of colonic manometry to guide surgical intervention has been reported in some series. Two series have described the use of manometry before and after formation of a diverting stoma, to aid in deciding whether further resection was required, or whether reversal of the stoma could be safely performed[231, 232].

It should be noted that the studies on children described here were performed using endoscopic catheter placement and, so, were short in duration and may have been affected by the use of sedative agents. In addition, this technique does not record simultaneously from all colonic regions. To date, no series of prolonged manometric studies of the whole colon, to allow the assessment of responses to meals, sleep, waking and the measurement of infrequent HAPS has been undertaken in children.

2.6 Medical management of simple constipation in children:

The management of simple constipation is achieved by a combination of dietary modification, behavioural therapy, rectal disimpaction, oral laxatives and, in some centres, biofeedback therapy.

2.6.1 Diet and behavioural modification:

An increase in fluid and fibre intake in order to avoid the passage of hard stools is usually recommended. However, dietary adherence in children is often difficult to achieve. Behavioural changes include instituting regular toileting and the use of stool frequency diaries[2].

2.6.2 Oral laxatives:

Stool softeners such as docusate sodium and lactulose are commonly used as first line oral laxatives. These are non-absorbable disaccharides, which increase intra-luminal water content. Polyethylene glycols (PEG, e.g. Movicol™, Kleanprep™, Golytely™, Colonlytely™) can be administered orally or via a naso-gastric tube, with a similar mechanism of action to sodium docusate and lactulose, but a more rapid onset. Adverse effects of these medications include bloating, abdominal cramps and flatulence[2, 206].

Oral stimulant laxatives are often used as a supplementary short-term adjunct to stool softeners. Examples of stimulant laxatives include senna (a type of anthraquinone), sodium picosulphate and bisacodyl. These laxatives work by a direct stimulatory action on colonic sensory nerves and activation of the myenteric plexus. They have an onset of action of 8-12 hours and adverse effects can include abdominal pain and incontinence[2, 206]. It should be noted that there are no randomised controlled trials to assess the efficacy of stimulant laxatives in the management of childhood constipation/soiling[233].

Stool lubricants, such as mineral oil, have been used successfully by some centres in the initial management of chronic constipation in children[206]. Bowel cleansing was achieved within 3-4 days and enemas avoided in nearly all cases. Reported adverse effects include pulmonary aspiration of lipid and malabsorption of fat-soluble vitamins[2, 206].

2.6.3 Rectal disimpaction:

The use of rectal suppositories and/or enemas to achieve initial emptying in children is controversial. Some centres deem this practice to be unacceptably intrusive[206]. However, the North American Society for Paediatric Gastroenterology and Nutrition recommend rectal disimpaction initially using either the oral or rectal route depending on the child's and parent's preference and then using oral laxatives as maintenance therapy[2]. Glycerin suppositories can be used in infants and bisacodyl suppositories in older children. For more severe cases, enemas such as phosphate or saline are effective. Enemas have the problem of non-compliance of the child and may also cause rectal irritation. However, they are quick and effective[2, 206]. In some children, rectal faecalomas develop to such a degree that oral or rectal medications are unlikely to allow evacuation of stool. Manual evacuation of the rectum under general anaesthetic is required in such instances.

2.6.4 Treatments to reduce anal sphincter hypertonicity:

It has been suggested that the symptoms of intractable constipation in a proportion of children might be associated with hypertonicity of the internal anal sphincter (section 2.5.2)[208]. This feature has been recorded manometrically, and is also called internal anal achalasia[234]. Several treatments directed at overcoming this hypertonicity have

been proposed. Anal dilatation or anal stretch has been employed, but this strategy remains somewhat controversial. If it is not carefully controlled, sphincter damage, which may be permanent, can occur[235]. Although lateral internal anal sphincterotomy may produce more precise sphincter division, this method is not in favour amongst some paediatric surgeons (in the context of anal fissure, for example)[236-238]. Internal anal myectomy has been used to treat intestinal neuronal dysplasia and short-segment Hirschsprung disease, but there are no studies describing this method to manage idiopathic constipation[166, 168, 169]. Recently, there has been some interest in the use of agents such as glyceryl trinitrate and the bacterial *clostridium botulinum* toxin to induce relaxation of the anal sphincter and so allow relief of symptoms in conditions such as (post-operative) Hirschsprung disease, chronic anal fissure and idiopathic constipation [234, 239, 240]. It will be interesting to see whether these innovations become established in the management of children with idiopathic chronic constipation.

2.6.5 Biofeedback therapy:

Biofeedback therapy involves the re-training of children in order to reverse the abnormal external anal sphincter contraction and decreased rectal sensation seen in many children with severe constipation, particularly those with functional faecal retention[8]. Benninga *et al*[8] reported that, in 29 children, external anal sphincter contraction on attempted defaecation was present in 55% and reduced rectal sensation present in 27%. After 5 biofeedback sessions using manometric measurements demonstrated to the children, 90% had normal external anal sphincter relaxation and 83% had normal rectal sensation. However, it should be noted that this technique is

expensive, time-consuming and requires a high degree of co-operation of the child and his/her family. In addition, relapse is a potential problem[8].

2.7 Surgical management of chronic constipation:

A range of techniques are available for the surgical management of constipation and faecal incontinence in children. These include establishing a continent appendix stoma, the formation of a non-continent colostomy or ileostomy, and partial or total colonic resection. The continent appendix stoma is a method of carrying out antegrade colonic washouts in children, first described in 1990[35]. Since this description, the emphasis of surgical management for constipation in children has shifted from major interventions such as colonic resections or colostomy formation, to minimally invasive methods aimed at allowing access to the colon to facilitate colonic lavage.

Malone *et al*[35] first reported the technique of reversing and re-implanting one end of the appendix into the caecum and siting the other end as a skin level stoma. A non-refluxing continent stoma was thus created, through which the proximal colon could be accessed and colonic lavage fluid instilled. This technique was named the 'antegrade continence enema' (ACE). Since this description, the technique has been modified and used worldwide to manage faecal incontinence secondary to causes such as Hirschsprung disease, ano-rectal malformations and spina bifida[36-38, 241]. Functional outcome using the ACE has been reported in relation to technical modifications, aetiology and patient selection.

Curry *et al*[37] reported a series of 31 children in whom the ACE procedure had been undertaken. Eight of these children had functional faecal retention and a success rate

of 39% was reported, compared to a success rate of 73% when used for other disorders. Meier *et al*[241] reported a 90% success rate in using the ACE to treat children with spina bifida or ano-rectal malformations, forming a reversed appendix stoma in 60% and non-reversed appendicostomy in 40%. Levitt *et al*[36] reported a series of 20 patients in whom a modified ACE procedure was undertaken to manage faecal incontinence, which in most cases was secondary to ano-rectal malformation. The modification described leaving the appendix *in situ* and plicating the caecum around the appendix to create a continent valve. It was reported that control of soiling was achieved in 95%.

Marshall *et al*[38] reported a series of 40 children who had undergone the ACE procedure for the treatment of slow-transit constipation. A successful outcome in relation to soiling was reported in 65%, however, again it was acknowledged that patient selection was an important factor in determining long-term benefit. A high rate of early complications was reported in this group, including stomal stenosis (55%), reflux of faeces (10%), reflux of mucous (69%), pain on washout administration (85%) and slow evacuation (41%). This emphasises the need to counsel patients and their families pre-operatively in order to gain long-term benefit.

Laparoscopic formation of the appendix stoma has been described [242, 243 244]. Low complication rates have been described using this technique for the management of faecal incontinence secondary to ano-rectal malformation, spina bifida and cerebral palsy[242, 244]. Lynch *et al*[243] reported a series of laparoscopic appendicostomy formations and compared their findings to other reported series describing open appendicostomy formation. A low rate of stomal reflux was reported (7%, compared

to up to 50% in open surgery) and control of soiling was achieved in 90%. It was concluded that laparoscopic formation of the appendix stoma was simple and safe.

Shandling and Chait *et al*[245] have described a radiological approach to provide access to the proximal colon for washouts. These authors have described percutaneous insertion of a catheter, under local anaesthetic, into the caecum using fluoroscopic control. The first report of this technique described the insertion of a gastrostomy device, which had a Foley-type balloon at the tip to secure the catheter in the caecum. In order to address the problem of balloon leakage and the unsightly appearance of the bulky catheter, the same authors developed a low-profile caecostomy catheter, which incorporates helical coils at the tip to hold the catheter in place[246, 247]. Reported complications of this technique included cellulitis, granulation tissue formation and a possible intraperitoneal leak of faecal content. In one case the caecum could not be accessed percutaneously due to overlying redundant sigmoid colon.

Gauderer *et al*[248] reported experience with placement of a catheter into the sigmoid colon in the management of children with distal hold-up of colonic content. They described a combination of laparoscopy and sigmoidoscopy to site a catheter in the left iliac fossa. The reported advantages of this technique were that a lower volume of washout fluid could be used as the whole colon was not irrigated, and that faster evacuation could be achieved by direct stimulation of the left colon. However, it was noted that potential disadvantages might include 'pulling' on the tube during sigmoid colonic contractions, and the formation of faecal fistula, rather than a catheterisable conduit. Haddad *et al*[249] have described the percutaneous insertion of a sigmoid irrigation tube using sigmoidoscopic guidance only.

Faecal diversion using either a colostomy or an ileostomy has been used in the management of intractable constipation. However, this is usually regarded as a 'last resort' due to the complications associated with stomas, as well as the cosmetic appearance. Villarreal *et al*[231] have reported use of a temporary diverting stoma in the treatment of chronic constipation, combined with subsequent colonic manometric investigation. It was suggested that further decisions regarding closure of stoma, or segmental colonic resection, could be directed on the basis of manometry findings. However, Nour *et al*[250] have reported on the complication rates associated with stomas in children. Complications occurred in 28% of a series of 138 children, these included prolapse, stenosis, retraction, parastomal hernia and skin-related problems.

Excision of part, or all, of the colon has been used to manage children with severe constipation who do not respond to conservative therapies and in whom measures such as the ACE procedure either have failed, or are likely to fail. There is little available evidence on functional outcome following colonic resection for intractable constipation in children. Godbole *et al*[33] have reported a small series of 3 children with idiopathic constipation and megarectum in which a staged Duhamel pull-through procedure was undertaken. Lee *et al*[34] reported a series of 4 children with intractable constipation and faecal incontinence in whom a rectosigmoid resection was undertaken at the same time as a temporary caecostomy was fashioned. Bowel function returned to normal in all 4 children and the caecostomy use was discontinued. Successful outcome was reported in this carefully selected group. Favourable results have also been reported in relation to resection of the dilated rectum and sigmoid colon in children with severe constipation secondary to ano-rectal malformations[251].

Colonic resection has been widely used in the treatment of adult slow-transit constipation[21, 252-254]. Several authors have reported outcome of these procedures and inferences can be made concerning the outcome of this approach in children. Stabile *et al*[252] reported 'mixed' results using partial colonic resection for idiopathic megarectum/megasigmoid. Of 7 patients treated, 1 died from post-operative intra-abdominal bleeding, 1 developed a rectovaginal fistula and 1 developed a pelvic abscess; the latter 2 required ileostomy formation. Lubowski *et al*[21] reported a success rate of approximately 75% using total colectomy. However, significant complications occurred, including anastomotic leak, intra-abdominal abscess and adhesive small bowel obstruction. Knowles *et al*[253] reported a review of 31 articles reporting outcome of surgery for constipation. An overall success rate of 90% was reported for subtotal colectomy. However, small bowel obstruction (18%), re-laparotomy for small bowel obstruction (14%) and permanent ileostomy formation (5%) were described. It was recommended that careful patient selection was required and that pre-operative investigation including physiology studies would improve outcome. As colectomy for slow-transit constipation may have similar complication rates in children, it is equally important to reserve this for 'last resort' cases. Suilleabhain *et al*[254] have reported use of colonic manometry to direct decisions regarding limited or total colonic resections in adults with slow-transit constipation and it is possible that this approach might be applicable to children.

3. Radio-isotope transit studies in the assessment of idiopathic chronic constipation in children.

The scintigraphic images were performed and interpreted by Dr. David Cook (Consultant Radiologist, Department of nuclear medicine, Royal Children's Hospital, Melbourne). Data on the 101 patients was collected by Mr. Benjamin Cook (Department of paediatric surgical research, Royal Children's Hospital). All of the text, including data analysis, the literature review, figure preparation and the discussion were undertaken by myself.

3.1 Introduction:

Idiopathic chronic constipation in children remains difficult to classify as there are no universally agreed definitions of normal or abnormal colonic transit in the paediatric population. Several studies have been reported in the medical literature in which gastro-intestinal transit has been measured in healthy children. Slow-transit constipation is a well-recognised disorder in adults, however, the equivalent disease is poorly described in children and large variations in the definition of slow colonic transit are used. For example, Benninga *et al*[13] have proposed a cut-off of delayed colonic transit beyond 100 hours to diagnose paediatric slow-transit constipation. This is in contrast with the findings of Bautista Casanovas *et al*[200], who reported the upper limit of normal transit in healthy children to be 50.2 hours (mean plus 2 standard deviations).

Early studies of gastro-intestinal transit involved the timed ingestion of non-absorbable markers, which were subsequently evacuated in the stool. The assessment of transit then progressed in sophistication with the introduction of radio-opaque

marker studies. Colonic regions of interest were assigned according to bony landmarks and colonic gas outlines seen on XR. This allowed estimates of transit time to be made with respect to the distribution of ingested markers. Some authors have advocated using different marker shapes ingested on different days or the use of multiple XRs to refine the assessment of transit. However, it has been noted that these additions to the technique do not necessarily improve the estimate of transit[200].

Scintigraphy is the measurement of the location of an ingested radioisotope as it transits through the gastro-intestinal tract. This method can be used to record gastric, small bowel and segmental colonic transit times without increasing radiation exposure. The geometric centre of the radioisotope at any given imaging time assists in the measurement of transit[18]. Scintigraphy is being used increasingly as a more accurate investigation for chronic constipation in adults,[19, 255, 256] but this technique has not been applied to children.

A review of the medical literature has demonstrated that 9 studies have reported the assessment of gastro-intestinal transit times in healthy children[10, 15, 16, 127, 196, 198-202]. A meta-analysis of these studies has been undertaken to determine an overall estimate of 'normal' colonic transit (section 2.6.1.4). Studies that described either a range or a mean with standard deviations were used to define an upper limit of normal (upper limit of the range described or the mean plus 2 standard deviations). Based on a total of 195 children studied, the mean colonic transit time in healthy children was found to be 29.9 hours with the upper limit of normal being 57.7 hours.

In this study, the results of 101 consecutive scintigraphic studies of children with idiopathic chronic constipation were reviewed retrospectively. The aim was to determine whether the advantages of colonic scintigraphy over radio-opaque markers noted in the assessment of adults also applied to children. In addition, the validity of combining visual interpretation of scintigraphic images with the calculation of the geometric centres of radioactivity in sub-classifying children with idiopathic chronic constipation was assessed.

3.2 Methods:

A retrospective review of 101 consecutive nuclear transit studies performed on children with severe constipation over a 2-year period was undertaken. All patients investigated had symptoms of severe, chronic constipation with or without soiling and had failed at least 6 months of aggressive medical therapy, with laxatives, dietary alterations and behavioural modification.

3.2.1. Nuclear Transit Study Protocol:

Laxatives were stopped five days before the transit studies and the patients fasted for four hours prior to the start of the test. Rectal disimpaction was not carried out prior to the study in any patients. No patients had obstructing faecalomas at the time of study. The radiopharmaceutical ^{99m}Tc-calcium phytate colloid, suspended in 20 mls of milk, was administered by mouth. The dose was determined according to the patient's weight and was based on an adult dose of 250 MBq. Images were acquired with a GE Starcam 3200 scintillation camera fitted with a low energy general-purpose collimator in a 128 x 128 matrix. Anterior and posterior view images were obtained immediately following ingestion and during the subsequent 2 hours, to estimate gastric emptying.

Following that series, the patients ate and drank normally. Images (anterior view only) were then acquired at 6 ± 1, 24 ± 2, 30 ± 2 and 48 ± 2 hours from the time of ingestion. Patients collected their faeces for quantification of faecal loss of radioactivity. Imaging was stopped at 48 hours, as limited information could be gained beyond this time due to radiation decay. The exposure to radiation for each patient (per study) was equivalent to between 1 and 2 plain abdominal XRs.

3.2.2. Visual Interpretation of Images:

The nuclear medicine radiologist at our hospital performed qualitative visual assessment of the images acquired at each time interval. Gastric emptying and small bowel transit were assessed in the images acquired up to 6 hours after ingestion of the radioisotope. Colonic transit was estimated by analysis of the images acquired between 6 and 48 hours. Studies were interpreted to assess whether recognisable patterns of transit were present.

3.2.3. Geometric Centre Analysis:

Six intestinal regions of interest were defined. These were region 1: the pre-colonic region; region 2: the caecum and ascending colon as far as the hepatic flexure; region 3: the transverse colon from the hepatic to the splenic flexure; region 4: the descending colon from the splenic flexure to the start of the sigmoid colon; region 5: the rectosigmoid colon and region 6: faeces. The decay and background-corrected activity in each segment were expressed as a percentage of the administered dose at each time of imaging. The estimates of faecal loss, by the counting of faecal radioactivity, were compared with the estimates obtained from the imaging of the abdomen. The geometric centre (GC) refers to the median point of the distribution of

activity within the colon and was calculated as described by Notghi *et al*[18]. The GC was calculated by multiplying the fraction of the administered activity in a region by the region number and the six numbers for each imaging episode were added. Thus, the distribution of activity in the colon was expressed as a single number for each time of imaging. The 4 calculated GCs corresponding to the distribution of activity at 6, 24, 30 and 48 hours were added to provide a single number which reflected the overall transit as measured during the whole study.

The individual GC for each time of imaging and the overall sum of the GCs, were compared with qualitative visual assessment of the serial images. Unpaired tests were used to compare the mean GC at each imaging time for the patterns of transit identified on visual interpretation.

3.3 Results:

Between October 1997 and October 1999, 101 consecutive patients were retrospectively included in the study, with 62 males and 39 females. The mean age at investigation was 7.3 ± standard deviation (SD) 3.7 years. All patients were referred with chronic constipation with or without soiling, which was resistant to more than 6 months of intensive medical therapy.

3.3.1 Qualitative Visual Assessment:

In 99/101 children the radioactivity passed from the mouth to the caecum or ascending colon within 6 hours. Two patients had studies suggesting impaired small bowel transit.

Three categories of colonic transit could be readily distinguished by visual assessment of the acquired images. In studies considered to demonstrate normal transit, tracer reached the caecum within 6 hours, passed through the colon and was excreted within 48 hours. A second group was appreciated in whom the tracer reached the rectosigmoid within 24-30 hours, but was not passed at 48 hours (Figure 1). This pattern was defined as being consistent with functional faecal retention (FFR) or outlet obstruction. Slow transit was identified when the tracer reached the caecum at 6 hours, but the majority of radioactivity was retained in the proximal and transverse colon at 24, 30 and 48 hours (Figure 2). Five studies showed a borderline picture, more like FFR than slow transit throughout the colon.

Employing these visual criteria, the 101 subjects were classified as follows: normal colonic transit in 24/101, slow colonic transit in 50/101, functional faecal retention in 22/101 and a borderline study in 5/101 children.

3.3.2 Assessment Using Geometric Centre of Activity:

Two of the 101 children had a GC of 1.0 at 6 hours, indicating that 100% of the tracer was located in the small bowel. In all other patients, at least part of the tracer was present in the caecum at 6 hours.

Analysis of the GC for the 3 visual patterns of transit showed that for those with qualitative normal colonic transit (24/101), the summed GC for the 4 imaging episodes was mean 15.7 ± SD 3.3. For the 50/101 patients with qualitative slow colonic transit, the sum of the 4 GC was 11.2 ± 1.9. For the 22/101 patients with functional faecal retention, the sum of the GC was 15.1 ± 1.5. The mean sum GC was

significantly lower ($p < 0.001$) in the patients with qualitatively slow colonic transit, compared to either those with qualitatively normal colonic transit or FFR (Table 2).

The mean, SD and range for the GC at each imaging episode in each transit pattern is shown in table 2. Statistical analysis of the GC at each of the imaging times showed that for the patients with qualitative slow colonic transit there was a significantly lower GC at 6 hours ($p < 0.05$), 24, 30, and 48 hours (all $p < 0.0001$), when compared to both those with qualitative normal transit and those with FFR (Figure 3). There was no significant difference in the GC at any imaging time when comparing those with qualitative normal transit to those with FFR (Figure 3). As demonstrated by the range of GC at each imaging time, there was no identifiable index that could be used alone to distinguish the 3 qualitative transit patterns at any imaging time.

3.4 Discussion:

The results of this study suggest that scintigraphy allows children with idiopathic chronic constipation to be classified into those with normal colonic transit, those with functional faecal retention and those with slow colonic transit. The advantages of scintigraphy over radio-opaque marker studies reported in adults also apply to the assessment of transit in children, as gastric and small bowel transit could be defined. In addition, the problem of ingesting multiple markers in children is avoided. In agreement with other studies reported on series of adults, we found that a combination of visual interpretation of acquired images at 6, 24, 30 and 48 hours and calculation of the geometric centres of radioactivity were required to define colonic transit.

It was found that approximately 2% of patients investigated for chronic constipation had previously unrecognised delayed small bowel transit, suggesting a pan-intestinal motility defect. The estimation of gastric and small bowel transit has been proposed as one of the advantages of scintigraphy over radio-opaque marker studies. This diagnosis has clinical significance, as this group will not benefit from colonic surgery. Stivland *et al*[17] noted delayed gastric emptying in 2 out of 8 constipated adults studied and delayed small bowel transit in a further 2 of these 8.

Visual assessment of the images acquired at 6, 24, 30 and 48 hours, together with the geometric centres of radioactivity, allows patients to be categorised into those with normal transit (24/101), those with slow transit (50/101) and those with FFR (22/101). This categorisation is clinically important, as these 3 groups require different treatment strategies. In cases of normal colonic transit, the tracer reaches the caecum in less than 6 hours and passes through the colon and is excreted by 48 hours. In patients with FFR, the tracer passes to the recto-sigmoid by 24 hours, but due to stool-withholding behaviour, it is not passed out of the rectum. Patients with slow colonic transit were identifiable by retention of tracer in the ascending and transverse colon at 30 and 48 hours.

The geometric centre of radioactivity appeared to reflect the categories of colonic transit determined on visual interpretation. Patients with qualitative slow-transit had significantly lower GC for each imaging time (6, 24, 30, 48 hours and sum of GC) when compared to those with FFR or normal transit. However, the GC is not sufficiently sensitive to be used in isolation as an index that can categorise colonic transit. As noted by Notghi *et al*[19], a GC of 4 (for example) at a particular imaging

time could mean that 50% of radioactivity is located in ROI 3 and 50% is located in ROI 5. Alternatively, a GC of 4 could also mean that 100% of the radioactivity is located in ROI 4. Thus, qualitative visual assessment of images acquired at each imaging time is essential to allow accurate interpretation of the GC.

The sub-classification of children with idiopathic chronic constipation has implications for treatment strategies. In FFR, transit as far as the rectosigmoid is normal. Therefore, once the rectum has been emptied (either with enemas or in severe cases with rectal disimpaction) and behavioural modification instituted, the condition should resolve. Insertion of a sigmoid irrigation tube[248] or even colonic resection[34] for FFR has been advocated in extreme cases by some authors. However, as the complication rate for colonic surgery can be high, this strategy remains controversial. Children who have reported symptoms of constipation, but normal colonic transit, are unlikely to benefit from surgical intervention. Instead, they require dietary alterations, laxatives and behavioural modifications. Children with slow transit require long-term management. These children may benefit from a high fibre diet[257], but most can be managed with laxatives and/or enemas. A proportion will not respond to conservative treatment and appendicostomy[38], colostomy or colonic resection[6] may be required. A proposed investigation/treatment algorithm for children with chronic constipation is shown in Figure 4.

In healthy adults, mean colonic transit has been reported as being between 26 and 43 hours using radio-opaque marker studies[12, 17, 196, 203, 258]. Using scintigraphy, mean colonic transit has been reported as 36 (+/- 6) hours in healthy adults[203]. Transit studies have been used to propose sub-groups of adults with chronic

constipation[18, 258]. Chaussade *et al*[258] reported transit in 91 adults with constipation (using radio-opaque markers), 49 had a normal colonic transit time, 16 had right colonic delay, 14 had left colonic delay and 12 had rectosigmoid hold-up. Notghi *et al*[18] described the use of scintigraphy to investigate 50 adults with constipation; 13 had normal colonic transit, 11 had generalised delay, 24 had right colonic delay and 2 had left colonic delay.

The time period required to assess colonic transit and thus diagnose slow-transit constipation in children is controversial. For example, Benninga *et al*[13] describe transit delayed beyond 100 hours as their definition for slow-transit constipation. However, Arhan *et al*[196] have reported that there is no significant difference in mean colonic transit between adults and children. Furthermore, a meta-analysis of studies which describe gastro-intestinal or colonic transit times in healthy children (undertaken for this thesis) suggests that the mean colonic transit time in children is 30 hours and the upper limit of normal is 58 hours[10, 15, 16, 127, 196, 198-202]. The range of reported mean colonic transit times was 18-76 hours.

Scintigraphy allows assessment of segmental colonic transit and so progress of tracer can be monitored during the study. Therefore, although imaging in the transit studies described in this report continues up to 48 hours, it is possible to interpret the progress of tracer through the colon at 24, 30 and 48 hours after ingestion and so define normal transit, slow transit and functional faecal retention. However, it is possible that the study protocol could be extended to 56-58 hours after ingestion of the tracer in borderline cases in whom the pattern of transit is not clear in the first 48 hours of the study.

In summary, this study indicates that scintigraphy allows accurate assessment of segmental colonic transit in children with unresponsive chronic constipation. The advantages of scintigraphy over radio-opaque marker studies, which have been reported in the investigation of adults with chronic constipation, apply to children. A combination of visual interpretation of acquired images and calculation of the geometric centres of radioactivity allows sub-classification of these children. Children with pan-intestinal motility disorders can be detected by the assessment of gastric and small bowel transit, which is possible with scintigraphy. Segmental colonic transit can be defined and so medical and surgical treatment strategies for normal colonic transit, slow colonic transit and functional faecal retention can be more accurately applied.

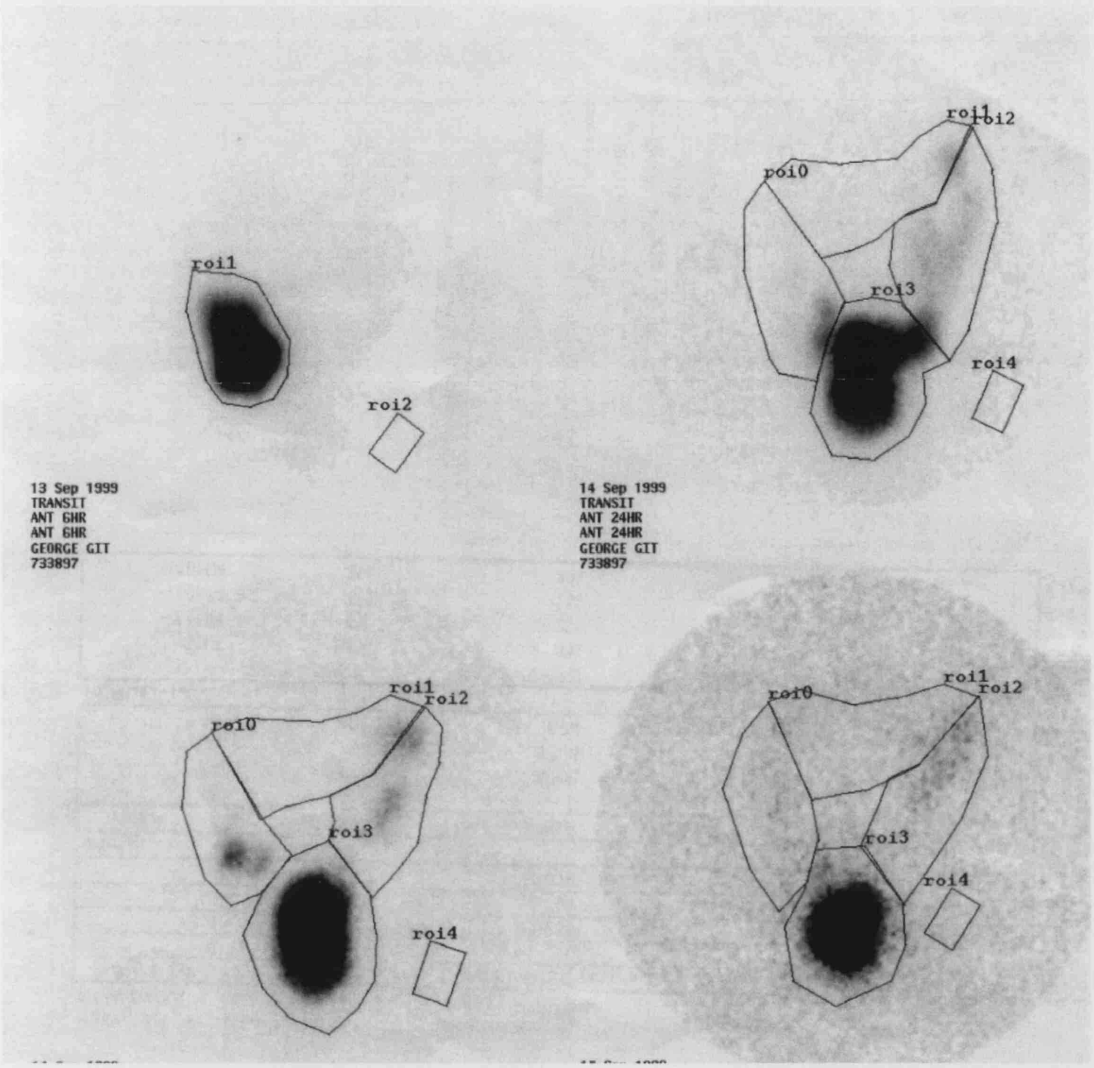

Figure 1: Example of a scintigraphic study showing functional faecal retention. Radioisotope transit is normal through the stomach and small bowel to the caecum (< 6 hours). Transit is also normal through the colon as far as the rectosigmoid at 24 and 30 hours. The tracer is retained in the rectum at 30 and 48 hours and not excreted.

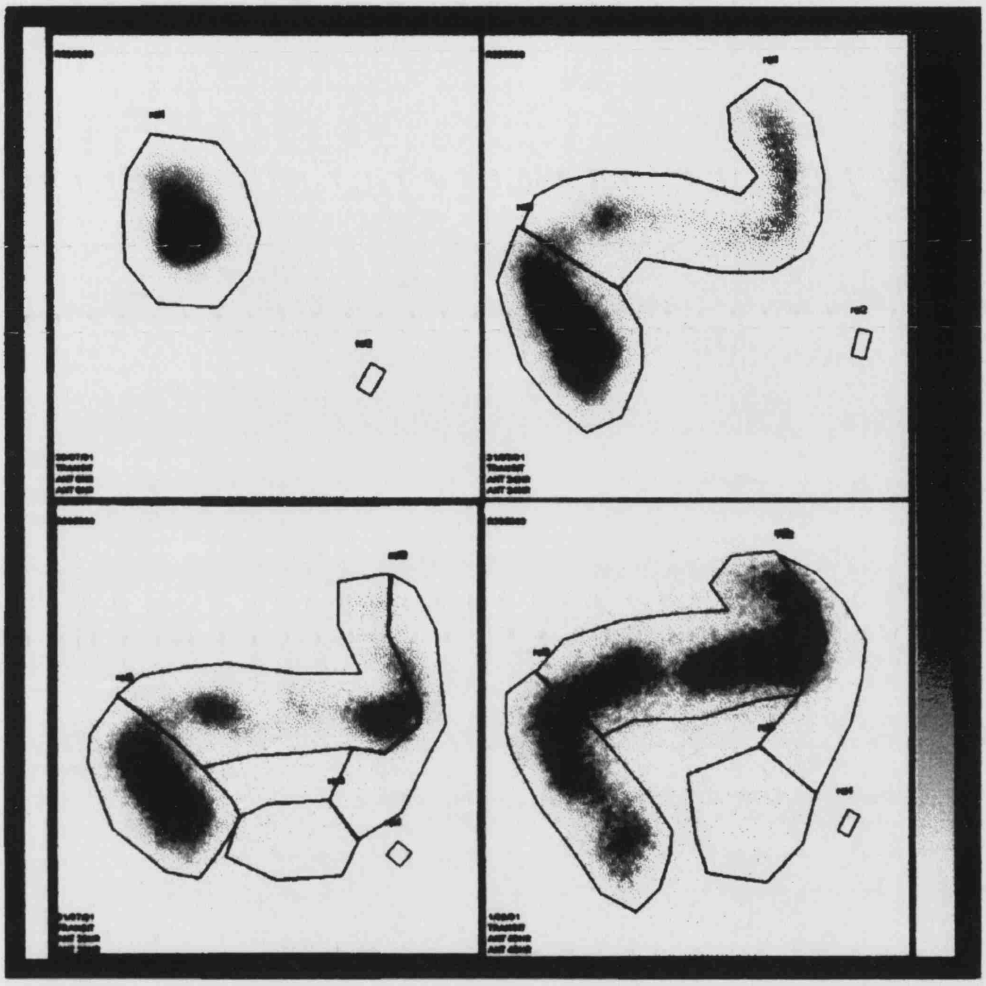

Figure 2: Example of a scintigraphic study showing slow colonic transit. Radioisotope transit is normal through the stomach and small bowel to the caecum. There is retention of the tracer in the right side of the colon at 24, 30 and 48 hours. The tracer has not reached the left side of the colon at 48 hours.

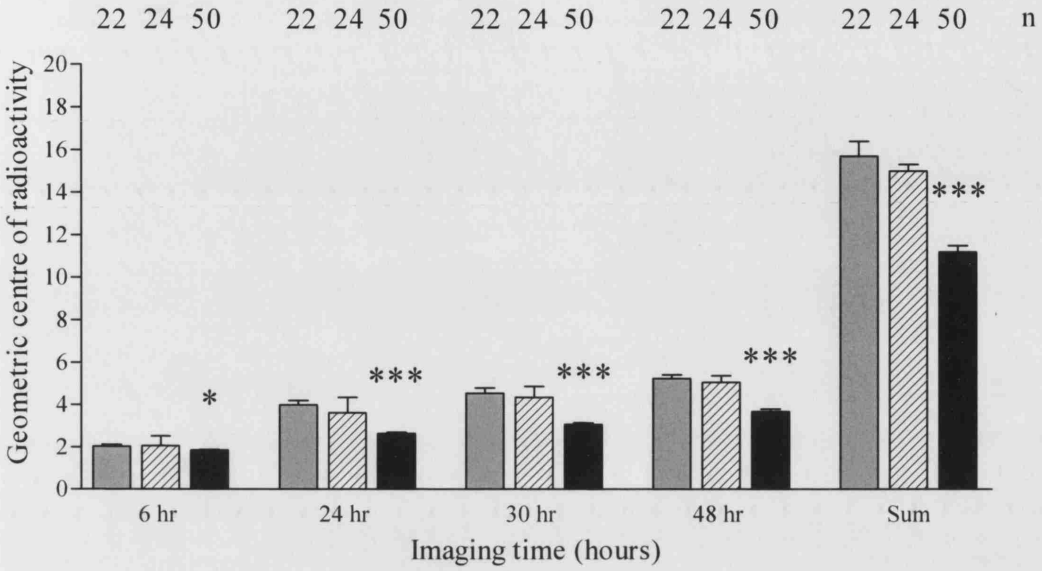

Figure 3: Geometric centres of radioactivity in 101 children with idiopathic chronic constipation. The geometric centres (GC) of radioactivity are shown for the 3 subgroups of transit determined using visual interpretation of images at 6, 24, 30 and 48 hours. The light bars are the 22 children with functional faecal retention, the striped bars are the 24 children with normal transit and the dark bars are the 50 children with slow colonic transit. The GC for the children showing slow colonic transit on visual analysis is significantly lower at 6 hours compared to those with normal transit and functional faecal retention (*$p < 0.05$). The GC for the children with slow colonic transit were also significantly lower at 24, 30 and 48 hours (***$p < 0.0001$) when compared to those with normal transit and functional faecal retention.

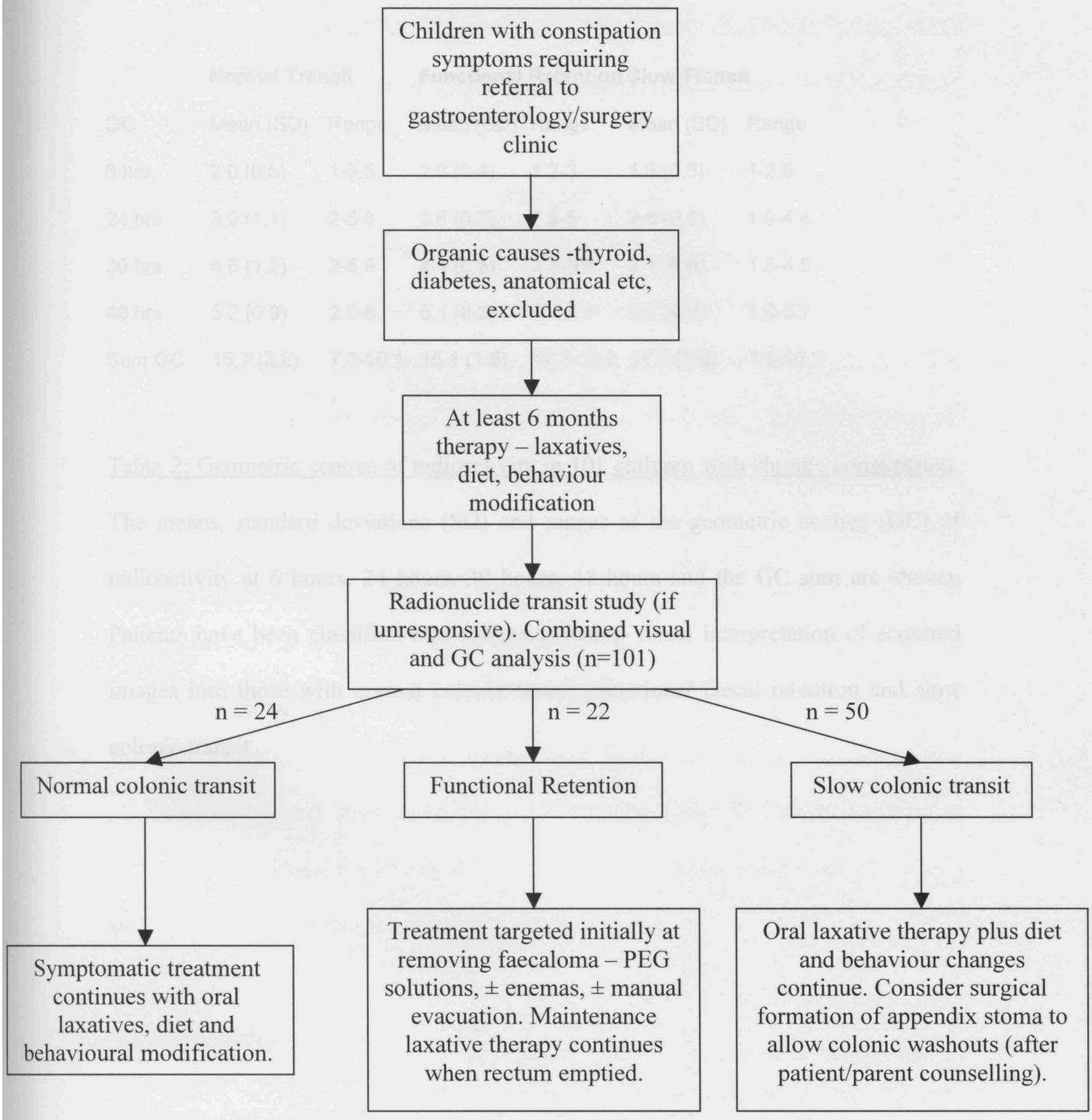

Figure 4: Algorithm for investigation and management of children presenting with symptoms of constipation. N = 24, 22, 50 refers to the number of patients with normal colonic transit, functional retention and slow colonic transit respectively, in the review of 101 patients who presented with constipation symptoms and underwent radionuclide transit study (5 patients had a 'borderline' study, more like functional retention than slow colonic transit).

	Normal Transit		Functional Retention		Slow Transit	
GC	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
6 hrs	2.0 (0.5)	1-3.5	2.0 (0.4)	1.2-3	1.8 (0.3)	1-2.5
24 hrs	3.9 (1.1)	2-5.9	3.6 (0.7)	2.5-5	2.6 (0.5)	1.9-4.4
30 hrs	4.6 (1.2)	2-5.9	4.4 (0.5)	3.5-5.4	3.1 (0.6)	1.8-4.5
48 hrs	5.2 (0.9)	2.3-6	5.1 (0.3)	4.4-5.7	3.7 (0.9)	1.9-5.7
Sum GC	15.7 (3.2)	7.3-19.1	15.1 (1.5)	12.7-18.2	11.2 (1.9)	7.5-16.3

Table 2: Geometric centres of radioactivity in 101 children with chronic constipation.

The means, standard deviations (SD) and ranges of the geometric centres (GC) of radioactivity at 6 hours, 24 hours, 30 hours, 48 hours and the GC sum are shown. Patients have been classified into subgroups using visual interpretation of acquired images into those with normal colonic transit, functional faecal retention and slow colonic transit.

4. Cholinergic transmission to colonic circular muscle of children with slow-transit constipation is unimpaired, but transmission via NK_2 receptors is lacking.

The first 24 (out of 74) muscle strip preparations described were carried out by Dr. Patricia Hengel (Department of surgical research, Royal Children's Hospital, Melbourne), using the same equipment and experimental technique. The immunofluorescence histochemistry was undertaken by Mrs Pamela Farmer, Dr. Bridget Southwell (Department of surgical research, Royal Children's Hospital, Melbourne) and Dr C W Chow (Department of pathology, Royal Children's Hospital, Melbourne). Confocal microscopy was performed by Dr. Bridget Southwell, Mrs Pamela Farmer and myself (Appendix 10.2). Data analysis of the immunohistochemistry studies and preparation of figures 17 and 18 were carried out by Dr. Bridget Southwell (Appendix 10.2). Data analysis of the physiology studies, the literature review, preparation of figures 5, 6 and 7, and the discussion were performed by myself. Professor John Carlin and Miss Gabrielle Davies (Department of biostatistics, Royal Children's Hospital, Melbourne) undertook statistical analyses of the data acquired from the physiology studies.

4.1 Introduction

The enteric nervous system controls gastrointestinal motility via several different functional classes of neurones including excitatory motor neurones, inhibitory motor neurones, sensory neurones and interneurones. The identities of these functional classes in humans have been inferred using a combination of retrograde labelling and fluorescence immunohistochemistry[69-72], although functional studies are yet to confirm many of these conclusions. The enzyme that synthesises acetylcholine (ACh),

choline acetyltransferase (ChAT), has been identified in just over 50% of all neurones projecting to the human colonic circular muscle (CCM), with the remaining neurones projecting to the CCM being immunoreactive for nitric oxide synthase (NOS). None of the NOS-immunoreactive neurones supplying the CCM are also immunoreactive for ChAT. The great majority (86%) of excitatory ACh-containing motor neurones project orally[71], while 77% NOS-immunoreactive neurones project anally. Of the orally projecting neurones that supply the CCM, 23% are immunoreactive for tachykinins (TK)[72], suggesting that many of the ChAT-immunoreactive neurones supplying the CCM also contain TKs. As ACh and TKs excite smooth muscle, while NO causes it to relax, the ChAT-immunoreactive neurones have been deduced to be excitatory motor neurones, while the NOS-immunoreactive neurones are assumed to be inhibitory motor neurones.

It has been assumed that ACh is the major excitatory neurotransmitter of the excitatory motor neurones supplying the CCM[29, 73]. However, recent studies have focussed on the contribution of TK in mediating excitation of the CCM, even though only a minority of the putative excitatory motor neurones has been shown to contain these peptides. Of the mammalian tachykinins, both substance P (SP) and neurokinin A (NKA) are thought to act in human colon, and all 3 major subtypes of TK receptors have been identified (NK_1, NK_2 and NK_3). Several studies have reported that the NK_2 receptor (for which NKA has the greatest affinity) is of primary importance in mediating tachykininergic excitation in human CCM[86, 88, 90]. Indeed, a recent study concluded that excitatory neuromuscular transmission in the human sigmoid colon is mediated entirely by TKs acting via NK_2 receptors, with there being no cholinergic component at all[30].

Identification of different types of motor neurones supplying the CCM has led to efforts to determine how such neurones may be involved in various motility disorders. Colon from patients with motility disorders has been studied in an attempt to describe changes in densities of motor neurones using immunohistochemistry, or deficits in functional neuromuscular transmission. For example, adult patients with slow-transit constipation (STC) have lower levels of TK-immunoreactivity (TK-IR) in their CCM[31, 172]. Tachykininergic neuromuscular transmission appears to be defective in adult STC[93] (almost all female), while cholinergic neuromuscular transmission has been reported as being either reduced or unaffected[77-80]

We have recently identified a population of children with STC, confirmed on radionuclide transit study[259]. These children differ from adult STC patients in being almost evenly divided between males and females. However, like the adult STC patients, many (63%) of these children have reduced TK-immunoreactivity (TK-IR) in their CCM on laparoscopic biopsy[6]. As ChAT and TK colocalise in human CCM motor neurones[71, 72], the question arises as to whether the reduced TK-IR indicated a deficit in excitatory neuromuscular transmission mediated by cholinergic neurones. Thus, the major aim of this study was to investigate excitatory neuromuscular transmission in paediatric STC and compare this with excitatory neuromuscular transmission recorded under identical conditions in colonic specimens taken from adults with normal colonic motility. Three questions were addressed. First, what are the roles of ACh and TK in excitatory neuromuscular transmission in adult CCM? Second, are these roles the same in CCM from children with STC? Third, does the finding of reduced TK-IR in a subgroup of children with STC correlate with a defect in either cholinergic and/or tachykininergic neuromuscular transmission?

4.2 Materials and methods:

4.2.1 Patient selection and specimen collection:

The tissue samples used in this study were obtained from two distinct patient populations. The first population was children with severe intractable constipation that had been unresponsive to at least 6 months of medical therapy (laxatives, enemas, behavioural modification) and who exhibited slow colonic transit on a radiolabelled ^{99m}-technetium study. Specimens containing longitudinal and circular muscle (but not mucosa or submucosa) of four colonic regions were obtained from each patient using standard 3-port laparoscopy. Specimens (5 mm x 3 mm) from the mid-transverse colon were used for the physiological studies described below, specimens from the right transverse colon, the left transverse colon and the sigmoid colon were used for immunohistochemical analyses.

The second population was adult patients undergoing colonic resection for carcinoma (irrespective of presence of chronic constipation). Specimens were taken as far away as possible from macroscopically tumour-involved tissue. Specimens for the physiological studies and for immunohistochemistry came from adjacent areas. Control specimens from healthy children were unavailable for obvious ethical reasons, so the adult specimens were used to provide a comparison with the paediatric specimens and to allow predictions from earlier studies to be examined.

Colon specimens were placed in ice-cold physiological saline (composition in mM: NaCl 118, KCl 4.8, NaH_2PO_4 1, $NaHCO_3$ 25, $MgSO_4$ 1.2, D-Glucose 11, $CaCl_2$ 2.5) within 15 minutes of excision for the paediatric specimens and within 60 minutes for adult specimens and transported to the laboratory. Specimens for

immunohistochemistry were fixed in Zamboni's fixative, while those for physiology studies were stored overnight at 4 °C. Initial experiments showed that overnight storage did not affect contractile responses (in agreement with other reports)[75].

Specimens of transverse CCM from a total of 37 STC children and 17 adults and sigmoid CCM from 20 adults were studied. The experimental protocol was approved by the Ethics-in-Human-Research Committee at the Royal Children's Hospital, Melbourne (Reference number 98072B).

4.2.2 Immunohistochemistry for substance P (tachykinin, TK):

The methods and results for the immunohistochemistry studies are described in Appendix 2. These results show that the pathologist's grading was able to distinguish between paediatric STC CCM with high and low SP when compared to confocal micrsocopy-assisted counting of SP-stained profiles. For physiology results, the hospital pathologist definition was used. Children graded as SP–2 or SP–3 were designated as 'low SP' and the rest as 'normal SP'.

4.2.3 In vitro pharmacology:

As the paediatric specimens lack mucosa and submucosa, these layers were removed from the adult specimens, which were then cut to produce 7 mm by 5 mm strips with their long axes parallel to the circular muscle. Laparoscopic specimens typically measured 5 mm by 3 mm and were oriented (by identifying the forceps marks that were parallel to the longitudinal muscle) so that circular muscle contractions could be measured.

The muscle strips were suspended in 6 ml organ baths containing physiological saline and attached to an isotonic transducer (SDR Technology, Biopac Systems, California, USA) with a resting tension of 0.3 g. The temperature in each organ bath was 37 °C, and the bathing physiological saline was bubbled continuously with 95%O_2/5% CO_2. The specimens were equilibrated for 1 hour, with changes of the bathing physiological saline every 10-15 minutes. Contractile responses were recorded onto a PC hard drive using a Biopac M100A (SDR Technology, Biopac Systems, California, USA) with a sampling rate of 10 samples/second and the data was analysed using Acqknowledge 3.2.4 software (SDR Technology, Biopac Systems, California, USA).

Carbachol (muscarinic agonist, 10 μM) was added to the organ bath first to elicit near-maximal contractions. Carbachol was washed out of the specimen by changing the bathing physiological saline every 5 minutes for 20 minutes. This served as a measure of the specimen's maximum capacity to shorten in response to a stimulus and was used to normalise the data across preparations. Contractions in response to NKA (100 nM) were elicited using the same protocol. Preliminary concentration-effect experiments showed that 10 μM carbachol (Appendix 1) and 100 nM NKA induced maximal contractions (Figure 6). Electrical field stimulation (EFS) was delivered to each preparation via 2 platinum plated electrodes placed on either side of the muscle strip parallel to the circular muscle. Stimulus pulses were triggered using a Grass S44 stimulator and an SIU5 isolation unit (Grass-Telefactor, Rhode Island, USA).

After washout of NKA, EFS (10 second trains of 0.2-0.6 ms duration, 60 V amplitude, 20 Hz frequency) was applied every 5 minutes, until a consistent response was seen. Typically this took 3-4 stimulations. This stimulus regime has been found

to produce large responses apparently mediated by tachykinins[30]. The effect of the following antagonists was assessed: SR 140333 (selective NK_1 receptor antagonist[88], 2 μM), SR 48968 (selective NK_2 receptor antagonist[88], 2 μM), SR 142801 (selective NK_3 receptor antagonist[89], 1 μM) and hyoscine (muscarinic antagonist, 2 μM). Previous reports indicated that the antagonist concentrations were great enough to achieve full receptor/neurotransmitter blockade[87, 88]. Incubation times of > 30 minutes were used for each antagonist prior to EFS; although SR 142801 typically requires a long incubation with lower concentrations, 1 μM produces full blockade within 30 minutes[260]. EFS contractions were measured immediately before addition of each antagonist, and after > 30 min incubation with antagonist. Antagonists were added cumulatively, both to save time and because preliminary experiments indicated that these compounds could not be washed out (results not shown). In most experiments the order of antagonist incubation was: SR 140333, SR 48968, SR 142801, hyoscine. However, in some experiments, this order was varied.

At the end of each experiment, the EFS response was re-tested in the presence of tetrodotoxin (TTX, 1 μM, 15 minute incubation), which specifically blocks neuronal action potentials, to demonstrate that contractions elicited by EFS were not due to direct stimulation of the muscle.

4.2.4 Data Analysis:

Contractions elicited by carbachol or NKA were measured in mm. EFS-induced contractions (also measured in mm) were expressed as a percentage of the contraction evoked by 10 μM carbachol in each specimen. The contraction evoked by EFS

immediately before addition of an antagonist served as the control for that antagonist. Statistical analyses were carried out by the Department of Biostatistics at the Royal Children's Hospital, Melbourne. Paired t tests were used to determine the significance of changes in EFS-induced contractions seen after antagonist incubation, for each specimen. Unpaired t tests were used to compare adult transverse with adult sigmoid CCM and to compare adult transverse with paediatric transverse STC CCM. The paediatric STC specimens were subdivided into those from colon with normal TK-IR and those with reduced TK-IR and unpaired t tests used for comparisons.

4.2.5 Chemicals Used:

Hyoscine and tetrodotoxin were purchased from Sigma-Aldrich, New South Wales, Australia. SR 140333, SR 48968 and SR 142801 were gifts from Sanofi Recherche, Paris, France. Neurokinin-A (ß-Ala^{8}-NKA-4-10) was purchased from Auspep Pty Ltd, Parkville, Victoria, Australia. All drugs were initially dissolved in distilled water to make stock solutions and then added as aliquots to the organ bath to give the desired final concentration in the organ bath.

4.3 Results:

The number and mean ages of STC children and adults are shown in Table 3. 65% of the STC children were assessed as having normal TK-IR tachykinin-immunoreactivity (TK-IR) in the transverse CCM (Table 3, Figures 17 and 18). Both carbachol and NKA elicited sustained contractions in circular muscle from adult transverse, adult sigmoid, and transverse colon from children with STC (Figure 5). The size of contractions elicited by both of these agonists was greater in the adult tissue than in the paediatric tissue. However, paediatric specimens (3 x 5 mm) obtained at laparoscopy were smaller than the adult specimens (5 x 7 mm) and when the contractions evoked by carbachol were normalised by dividing by the tissue length there was no apparent difference between paediatric and adult specimens. Contractions evoked by carbachol were 2 – 3 times larger than those evoked by NKA in adult transverse CCM (mean ± standard error of mean, SEM, 1.8 ± 0.2 mm and 0.6 ± 0.2 mm, respectively, $p < 0.001$, $n = 10$), in adult sigmoid CCM (1.5 ± 0.2 mm and 0.6 ± 0.1 mm, respectively, $p < 0.05$, $n = 9$) and in paediatric STC CCM (1.0 ± 0.2 mm and 0.6 ± 0.2 mm, respectively, $p < 0.05$, $n = 18$).

The ratio of NKA-evoked contraction to carbachol-evoked contraction (Figure 5B) was the same for circular muscle from adult transverse and adult sigmoid colon ($p > 0.3$) and from adult transverse colon and transverse colon from children with STC ($p > 0.9$). Similarly, this ratio did not differ significantly between circular muscle preparations from transverse colons with normal and reduced TK-IR from children with STC ($p > 0.1$).

4.3.1 Responses to electric field stimulation (EFS):

EFS evoked contractions in all preparations (Figure 6A). These were preceded by small relaxations in some specimens. In all cases, the contractions evoked by EFS were less than half the amplitude of those evoked by carbachol with the ratios being about 0.25 (adult transverse), 0.40 (adult sigmoid) and 0.25 (paediatric transverse CCM). Responses to EFS were similar in the two populations of paediatric STC specimens, 23 ± 3% (TK-IR normal, n = 24) and 27 ± 6% (TK-IR low, n = 13).

The muscarinic antagonist hyoscine (2 μM) greatly reduced EFS-induced contractions in all 4 types of colonic circular muscle - adult transverse, adult sigmoid, and STC paediatric transverse (Figure 6). This was true, even when the order of incubation of antagonists was altered. In 15 adult transverse colon specimens, mean EFS-induced contractions before hyoscine were 23 ± 3%, and were reduced significantly ($p < 0.001$) to 13 ± 3% after > 30 min of hyoscine incubation. In 16 adult sigmoid colon specimens, mean EFS-induced contractions before hyoscine incubation were 41 ± 6%, and were reduced significantly ($p < 0.0001$) to a mean of 15 ± 4% in hyoscine.

In transverse colon specimens from 30 children with STC, EFS-induced contractions were reduced significantly ($p < 0.0001$) from 28 ± 4% before hyoscine incubation, to 16 ± 3% after incubation in the muscarinic antagonist. In the subgroups of normal TK-IR and low TK-IR transverse colon, EFS-induced contractions were reduced significantly in hyoscine from 30 ± 6% to 19 ± 2% ($p < 0.005$) and from 25 ± 4% to 10 ± 4% ($p < 0.05$) respectively. The effects of hyoscine on EFS-evoked contractions did not differ significantly between the two groups of paediatric STC specimens ($p > 0.1$).

Sequential blockade of TK receptors was measured only in transverse colon. Of the TK receptor antagonists, only the NK_2 receptor antagonist had significant effects on contractions evoked by EFS (Figure 7). In the presence of the NK_2 receptor antagonist SR 48968 (2 μM), EFS-induced contractions were reduced by about $1/5^{th}$ ($p < 0.05$) in adult transverse CCM (n = 12) from 34 ± 6% to 28 ± 6% (Figure 7C, paired t test). In contrast, in paediatric STC CCM (n = 29) responses were unaffected by the NK_2 receptor antagonist SR 48968. The subgroups of normal TK-IR and low TK-IR were similarly unaffected by SR 48968 (Figure 7C).

Incubation with the NK_1 receptor antagonist SR 140333 (1 μM) resulted in a small non-significant increase in EFS-induced contractions in adult and paediatric STC transverse CCM (Figure 7B). In the presence of the NK3 receptor antagonist SR 142801 (1 μM), there was a small non-significant reduction in EFS-induced contractions in adult and paediatric STC child transverse CCM (Figure 7D). The subgroups of TK-IR normal and TK-IR reduced were similarly unaffected by either SR 140333 or SR 142801 (Figure 7B, 7D).

TTX (1 μM) virtually abolished the contractions evoked by EFS in all preparations (Figure 7A) indicating that these contractions were not due to direct excitation of the muscle by the electrical stimulus.

4.4 Discussion:

4.4.1 Cholinergic and tachykininergic transmission in adult colonic circular muscle:

These results confirm that ACh is the major neurotransmitter mediating contraction of human adult and paediatric colon. They also confirm that cholinergic neuromuscular transmission is unimpaired in the transverse colon of children with STC, whether they have normal or low TKIR. Thus, although TK and ChAT are co-localised in CCM excitatory motor neurones, low TK-IR is not a marker for impaired cholinergic transmission in paediatric STC. This conclusion is supported by the finding that the muscarinic antagonist hyoscine significantly reduced EFS-induced contractions in all types of CCM (by 35 – 80%) and this reduction was similar in transverse CCM from adults and STC children with either normal TK-IR or low TK-IR. The muscarinic agonist carbachol (10 μM) induced large contractions of 1-2 mm in adult transverse CCM, adult sigmoid CCM and transverse CCM from children with STC.

That ACh mediates neuromuscular transmission in adult human CCM was reported nearly 30 years ago[29]. More recently, tachykininergic neuromuscular transmission in adult human colon has also been reported[90, 91]. This appears to be mediated by NK_2 receptors on the circular muscle[88].

We also identified a tachykininergic component of excitatory neuromuscular transmission in adult transverse CCM. This was mediated by NK_2 receptors, but not by NK_1 or NK_3 receptors. NKA (which has greatest affinity for NK_2 receptors) elicited contractions approximately $1/3^{rd}$ the size of those elicited by carbachol. In adult colon, EFS-induced contractions were significantly reduced by about $1/5^{th}$ by

NK_2 receptor blockade, but were unaffected by NK_1 or NK_3 receptor antagonism as has been observed by others[88, 90, 91]. The proportional reduction by muscarinic blockade was substantially greater (about ½ of the control) than that produced by the NK_2 antagonist. This suggests that ACh is the major excitatory neurotransmitter in human adult CCM, while NKA (acting via NK_2 receptors) has a smaller role.

In contrast to these observations, Cao *et al*[30] reported that isotonic contractions induced by EFS in human sigmoid colon were unaffected by the muscarinic antagonist atropine (100 μM), but were abolished by NK_2 receptor. This was surprising, as 50% of nerve fibres in human CCM contain ChAT[70] and ACh is the major excitatory neurotransmitter in guinea pig, rat, dog and pig colon. The disparity may be due to our use of longer incubation times before testing the muscarinic antagonist's effect (> 30 minutes compared to 15 minutes by Cao *et al*[30]). In both adult and paediatric specimens, a tetrodotoxin-sensitive contraction was evoked by EFS in the presence of supramaximal concentrations of both muscarinic and NK_2 receptor antagonists. Thus, another, as yet unidentified, neurotransmitter/receptor combination may mediate a component of excitatory neuromuscular transmission in human colon.

4.4.2 Tachykininergic transmission in colonic circular muscle from STC children:

In children with STC, NKA evoked contractions in transverse CCM that were about half the size of those evoked by the muscarinic agonist carbachol. Similar contractions were seen in CCM with normal TK-IR or low TK-IR. This suggests that functional TK receptors are present in both subgroups of children with STC and that

upregulation of receptors does not occur. NK_1 and NK_3 receptor antagonism produced no change in EFS-induced contractions in CCM from STC children. In contrast to adult transverse CCM, NK_2 receptor blockade did not alter EFS-induced contractions, whether TK-IR was normal or low. Thus, functional NK_2 receptors are present, but TKs do not appear to mediate excitatory neuromuscular transmission in children with STC. It is not clear if this is related to constipation or to childhood. Unfortunately, we do not know the timing of development and maturation of TK transmission in normal colon. Establishing the status of TK transmission in colon from children without gastro-intestinal anomalies is difficult as seromuscular colonic biopsies cannot be obtained from healthy children for ethical reasons and colonic resections are rarely undertaken in children for pathological processes. In this study, adult colon was the only tissue available to use for comparison. It will, however, be important to study changes in TK transmission during childhood.

There have been no previous studies of neuromuscular transmission in CCM from children with STC, but CCM from adults with STC has been examined in several studies. Slater *et al*[77] reported that the muscarinic agonist carbachol elicited larger contractions in adult STC CCM than in controls and proposed that there was 'denervation hypersensitivity' in response to impaired cholinergic neurotransmission. Burleigh *et al*[78] found a decrease in the release of radiolabelled ACh when colonic longitudinal muscle strips were stimulated electrically. This is consistent with a study using multipoint colonic manometry to show impaired responses to cholinergic stimulation in the descending colon of adults with STC[79]. However, Mitolo-Chieppa *et al*[93] found that atropine reduced EFS-induced frequency-tension curves to the same extent in colon from adults with idiopathic chronic constipation and colon from

controls, indicating that there was no abnormality. There is also controversy concerning tachykininergic neurotransmission in colon from STC adults. Tachykinin receptors have been reported to increase[92] or decrease[93] in CCM from such patients.

The relationship between paediatric and adult STC is currently unknown. It is unlikely that the 2 disorders have the same aetiology, despite being similar in clinical presentation. The gender ratio in childhood STC is approximately 50:50[6], whereas virtually all adult STC patients are female.

Porter *et al*[31] found no difference in cholinergic immunoreactivity in CCM from adults with STC and CCM from adult age-matched controls; however, TK-IR was significantly lower in CCM of adults with STC than in controls. Low SP-IR has also been reported in the mucosa and submucosa of rectal biopsies from adults with STC[261]. TK-IR is low in the CCM of approximately 65% of STC children[172]. It is unclear whether alterations in TK-IR in adult and/or childhood STC are a primary phenomenon, or are secondary to long-standing constipation or treatment regimens. However, Tzavella *et al*[262] have reported that long-term treatment with laxatives did not affect TK-IR in rat distal colon.

This raises the question of whether a reduction in the release of TKs from motor nerve terminals or elsewhere could be expected to produce a substantial deficit in colonic transit. In guinea pig ileum and colon, NK_1 receptors are located on muscle cells, interstitial cells of Cajal, inhibitory neurones and sensory neurones, NK_2 receptors are found on muscle cells, and NK_3 receptors on excitatory neurones, interneurones and sensory neurones[82-85, 263-265]. Intrinsic sensory neurones and ascending

interneurones contain TKs[266] and contact many neurones that express the receptors. TKs are widely distributed within the human colon[267] and TK receptors are probably located on both neuronal and non-neuronal sites in the human. Thus, a TK deficiency may have its main effect at sites other than the muscle. However, the available specimens were too small to allow study of other sites of action within the enteric neural circuitry.

4.5 Summary:

ACh-mediated neuromuscular transmission is present in paediatric STC, even when TK-IR is low. Tachykininergic neuromuscular transmission occurs in adult CCM via NK_2 receptors, but not in paediatric STC CCM, despite the presence of functional TK receptors. This failure of tachykininergic neuromuscular transmission is seen in paediatric STC CCM with both normal and low TK-IR.

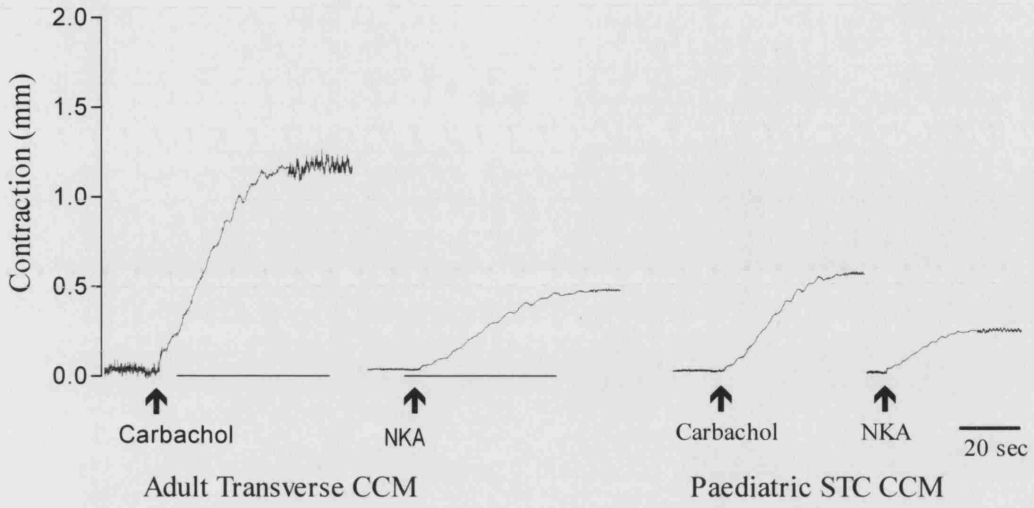

Figure 5 A: Carbachol and neurokinin A induce contraction of colonic circular muscle. Example traces showing contraction induced by 10 μM carbachol and 100 nM NKA in colonic circular muscle from an adult (colon resected for carcinoma, Adult Trans) and from a child with slow-transit constipation (Paed STC).

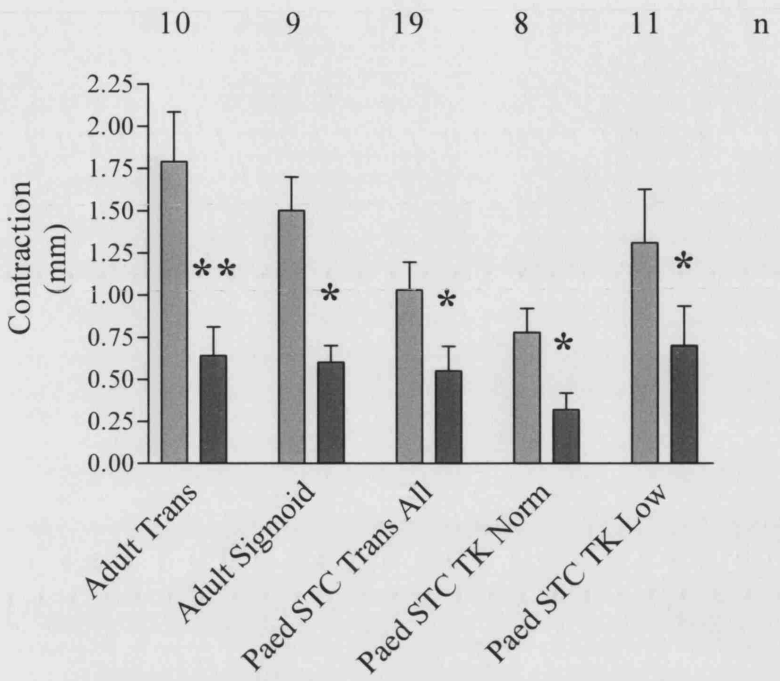

Figure 5 B: Mean contractions of colonic circular muscle in response to carbachol and neurokinin A. Contractions (mean ± SEM) induced by 10 μM carbachol (light bars) and 100 nM NKA (dark bars) in transverse colonic circular muscle from adults (carcinoma patients) and children with slow-transit constipation. Paed STC Trans All indicates all specimens taken from children with STC and data from the two subdivisions of these patients, those with normal TK-IR (Paed STC TK Norm) and those with low TK-IR (Paed STC TK Low) are also shown separately. Numbers above each pair of columns indicate the number of specimens tested. * indicates $p < 0.05$ (comparing carbachol-induced contractions to NKA-induced contractions).

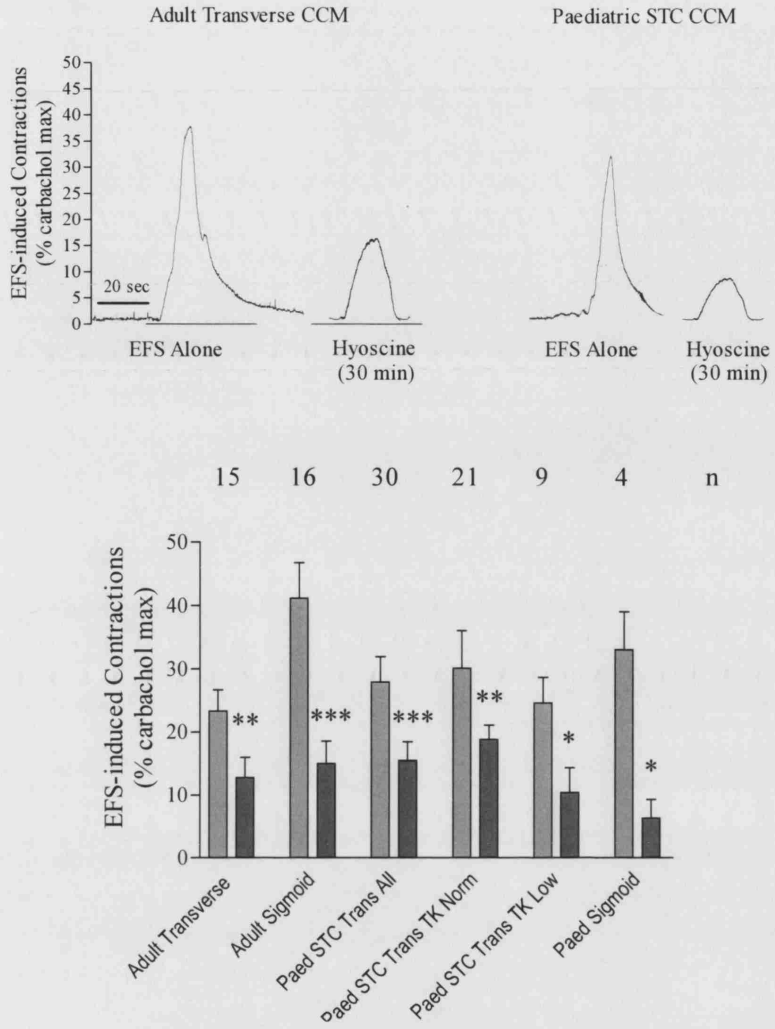

Figure 6: Acetylcholine is the major excitatory transmitter in colonic circular muscle.

A) Example traces of contractions induced by EFS before and after > 30 min incubation with the muscarinic antagonist hyoscine (hyos, 2 μM).

B) Mean contraction of colonic circular muscle in response to EFS before (light bars) and after incubation with 2 μM hyoscine (dark bars). Numbers in each pair of bars indicate the number of specimens tested; note this was less than the total sample for technical reasons. Contractions induced by EFS are significantly reduced by incubation with hyoscine in all types of CCM. Column labels are as for Figure 5. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, comparing EFS pre and post hyoscine, paired t test).

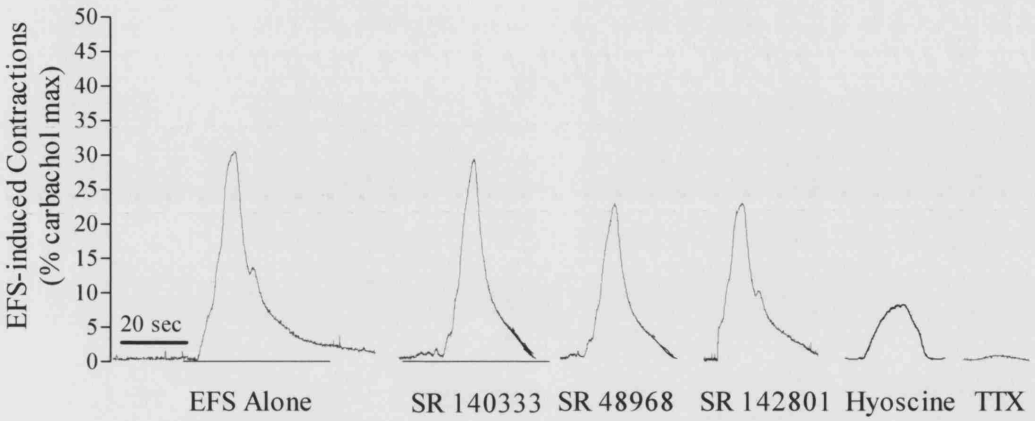

Figure 7A): Effect of tachykinin antagonists on contraction of colonic circular muscle.

Example tracings of contractions induced by EFS in colonic circular muscle from a child with slow-transit constipation with cumulative incubation (> 30 min each) of 1 μM SR 140333 (NK_1 receptor antagonist), 2 μM SR 48968 (NK_2 receptor antagonist), 1 μM SR 142801 (NK_3 receptor blockade), 2 μM hyoscine and 1 μM TTX.

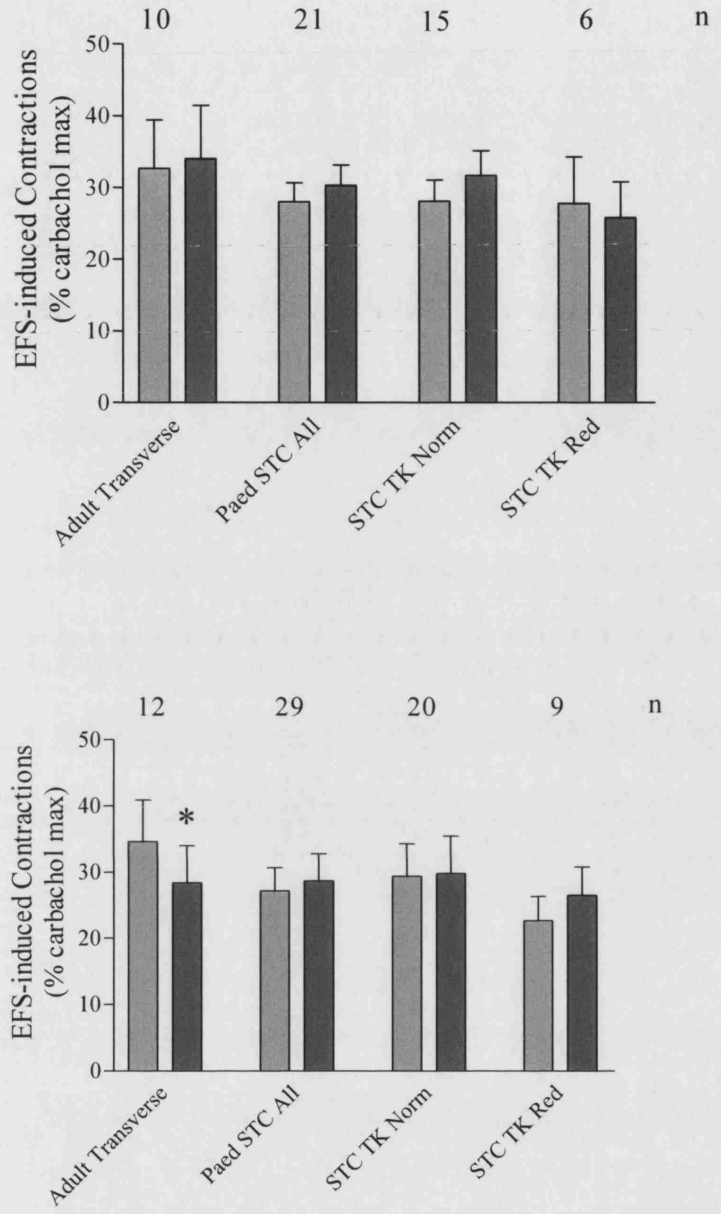

Figure 7 B-C): Effect of tachykinin antagonists on contraction of colonic circular muscle. Mean contractions in response to EFS before and after incubation with B) NK_1 receptor antagonist C), NK_2 receptor antagonist in colon from adults (carcinoma patients) and in colon from children with slow-transit constipation. Light bars indicated responses induced by EFS before antagonist incubation and dark bars indicate responses induced by EFS after > 30min antagonist incubation. Column labels are as for Figure 5 (* $p < 0.05$, comparing pre and post antagonist responses).

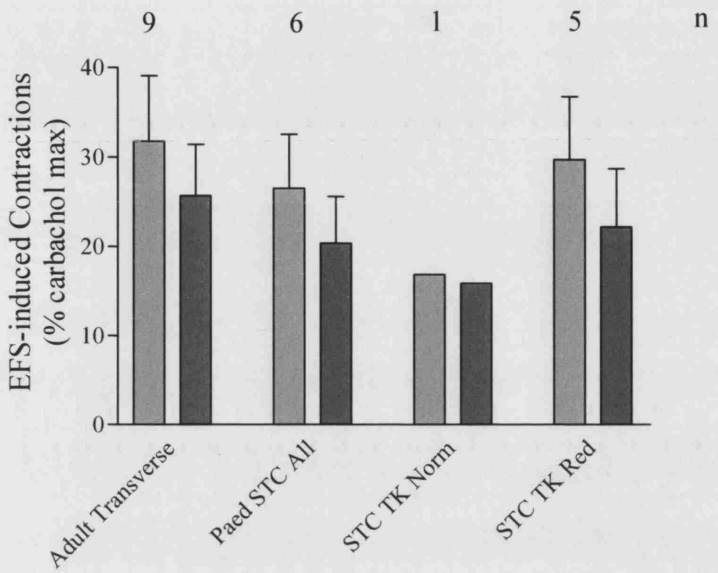

Figure 7 D): Effect of tachykinin antagonists on contraction of colonic circular muscle. Mean contractions in response to EFS before and after incubation with NK_3 receptor antagonists in colon from adults (carcinoma patients) and in colon from children with slow-transit constipation. Light bars indicated responses induced by EFS before antagonist incubation and dark bars indicate responses induced by EFS after > 30min antagonist incubation. Column labels are as for Figure 5 (* $p < 0.05$, comparing pre and post antagonist responses).

Patients-region	N	Age (yr)		
		mean	SD	Range
Adult				
Transverse Colon	17	66.2	17.6	23-86
Sigmoid Colon	20	68.3	13.1	41-87
STC Children				
Transverse Colon	37	8.7	3.7	2-18
Normal TK-IR	24	9.1	4.1	2-18
Low TK-IR	13	8.1	2.7	4-13

Table 3. Ages (mean standard deviation, SD and range) of patients and number (N) of samples of transverse and sigmoid colon from adults and from children with slow-transit constipation.

5. Inhibition and relaxation in colonic circular muscle of children with slow-transit constipation.

5.1 Introduction:

It was recognised by Bayliss and Starling that the phenomenon of inhibition/relaxation in intestinal circular muscle was as important as excitation/contraction in allowing antegrade propulsion of intestinal content. The 'law of the intestine' was applied to this reflex of ascending excitation/contraction and descending inhibitory/relaxation[22]. Functional and histological studies have demonstrated that nitric oxide is the predominant neurotransmitter mediating inhibitory/relaxation in human colonic circular muscle (CCM), possibly with vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP) having a lesser role[75, 94-96, 98].

Colonic muscle from patients with disorders of colonic motility has been studied in order to determine whether a defect in nitric oxide mediated inhibition/relaxation might be present. Failure of relaxation of colonic muscle has been reported in association with clinical scenarios such as Hirschsprung disease and adult idiopathic chronic constipation[109-111].

The aims of this preliminary study were, first, to assess whether relaxation responses could be elicited in strips of colonic circular muscle to provide a basis for investigating inhibitory neuromuscular transmission. The second aim was to study the

role of nitric oxide in mediating inhibitory neuromuscular transmission in adult CCM and CCM from children with slow-transit constipation.

5.2 Materials and methods:

Specimens of sigmoid CCM from 3 adults (carcinoma patients) and transverse CCM from 7 children with STC were used. It was not possible to increase the sample size (or to obtain adult transverse CCM specimens) within the resources available. Patient selection, specimen collection/transportation and experimental set-up were identical to that described for the *in vitro* contractility studies on cholinergic and tachykininergic neurotransmission (section 4). Immunofluorescence staining for putatative inhibitory neurotransmitters was not known for either the adults or the children with STC.

5.2.1 Relaxation Studies:

Sodium nitroprusside (SNP, nitric oxide donor, 1 μM) was added to the organ bath first, to elicit near-maximal relaxations. SNP was washed out of the specimen by flushing the bathing physiological saline every 5 min for at least 20 min. Relaxations evoked by SNP were repeated until a consistent maximal response occurred. Relaxation responses to EFS were divided by the magnitude of the response to SNP to allow comparisons between different preparations, as the SNP relaxation was taken to represent the largest response that could be produced by each preparation. Relaxations in response to isoprenaline (sympathetic agonist, 1 μM) were elicited using the same protocol as for SNP. Preliminary concentration-effect experiments showed that 1 μM SNP and 1 μM isoprenaline induced maximal relaxations. Electrical field stimulation (EFS) was delivered in an identical fashion to that used in the contractility studies.

After washout of isoprenaline, EFS (5-10 sec trains of 1.0 msec duration, 60 V amplitude, 10 Hz frequency) was applied every 5 min, until a consistent response was seen. Typically this took 3-4 repetitions.

The effect of Nitro-L-arginine (NOLA, nitric oxide synthase inhibitor, 100 μM) was assessed. Previous reports indicated that 100 μM NOLA was a concentration great enough to achieve full receptor/neurotransmitter blockade[87, 88]. NOLA was incubated for > 15 min prior to EFS. EFS contractions were measured immediately before addition of NOLA, and after > 15 min incubation with NOLA.

At the end of each experiment, the EFS response was re-tested in the presence of tetrodotoxin (1 μM, 15 min incubation), to demonstrate that contractions elicited by EFS were not due to direct stimulation of the muscle.

5.2.3 Data Analysis:

Contractions elicited by SNP and isoprenaline were measured in mm. EFS-induced relaxations (also measured in mm) were expressed as a percentage of the relaxation induced by 1 μM SNP for each specimen. The relaxation evoked by EFS immediately before addition of an antagonist served as the control for the effects of NOLA on neuromuscular transmission.

5.2.4 Chemicals Used:

SNP, isoprenaline and NOLA were manufactured by Sigma-Aldrich, New South Wales, Australia. All drugs were initially dissolved in distilled water to make stock

solutions and then added as specific aliquots to the organ bath to give the required final concentration in the organ bath.

5.3 Results:

The mean age of the 7 children with slow-transit constipation was 6 ± 2.1 SD years (range 4-9 years). The mean age of the 3 adult patients (sigmoid CCM) was 71 ± 5.5 years (66-77 years).

Both sodium nitroprusside (1 μM) and isoprenaline (1 μM) elicited sustained relaxations in CCM from adult sigmoid colon and CCM from transverse colon from children with slow-transit constipation (Figure 8). The size of relaxations elicited by both of these agonists was similar in the adult tissue and the paediatric tissue (Figure 10). Relaxations evoked by SNP were 2 – 3 times larger than those evoked by isoprenaline in adult sigmoid CCM (mean ± SEM, 0.4 ± 0.1 mm and 0.07 ± 0.05 mm, respectively, n = 3) and in paediatric STC transverse CCM (0.3 ± 0.1 mm and 0.04 ± 0.01 mm, respectively, n = 7). The ratio of SNP-evoked contraction to isoprenaline-evoked contraction was similar for colonic circular muscle from adult sigmoid colon and transverse colon from children with STC.

EFS evoked relaxations in all preparations (Figure 9); these were followed by contractions in most specimens (as seen in the contractility studies). In the adult sigmoid CCM, the relaxations induced by EFS were 30 ± 21% of the relaxations evoked by SNP (n = 3), while the equivalent values in the paediatric STC transverse CCM were 41 ± 9% (n = 7).

NOLA (100 μM) greatly reduced EFS-induced relaxations in adult sigmoid CCM, and reduced EFS-induced relaxations to a lesser degree in paediatric STC transverse CCM (Figure 10). In 3 adult sigmoid colon specimens, mean EFS-induced relaxations before NOLA were 33 ± 12%, and were reduced to 4 ± 6% after > 15 min of NOLA incubation. In transverse colon specimens from 7 children with STC, EFS-induced relaxations were reduced from 47 ± 10% before NOLA incubation, to 26 ± 9% after NOLA incubation.

TTX (1 μM) virtually abolished the relaxations evoked by EFS in all preparations indicating that these relaxations were not due to direct stimulation of the muscle.

5.4 Discussion:

In both transverse CCM from children with STC and sigmoid CCM removed from adults with carcinoma sodium nitroprusside (SNP, nitric oxide donor) and isoprenaline (sympathetic agonist) elicited relaxation responses. As relaxations were 2-3 times larger in response to SNP than in response to isoprenaline, it is probable that nitric oxide has a larger role in mediating inhibitory neuromuscular relaxation than sympathetic nerves. Electrical field stimulation elicited relaxation responses if a higher pulse duration (1.0 msec) was used than for the contractility studies (where typically 0.2 –0.6 msec was used).

In adult sigmoid CCM, relaxation responses induced by electrical field stimulation were almost entirely abolished in the presence of NOLA. NOLA is an antagonist for nitric oxide synthase, the enzyme that synthesises nitric oxide for L-arginine [100]. EFS-induced relaxations were reduced to approximately 10% of the maximal

relaxation induced by sodium nitroprusside. This suggests that, in adult sigmoid CCM, inhibition/relaxation is mediated predominantly by nitric oxide. It appears that other putatative neurotransmitters such as vasoactive intestinal peptide and adenosine triphosphate may, therefore, have a lesser role.

In transverse CCM from 7 children with slow-transit constipation, EFS-induced relaxations were reduced by approximately 50% in the presence of NOLA. This implies that, in paediatric STC transverse CCM, nitric oxide has a role in mediating neuromuscular relaxation, but that other neurotransmitters are also involved to a much greater degree than in adult sigmoid CCM. This is a potentially significant finding, if confirmed in a larger series.

Both muscle strip contractility studies similar to this one and electrophysiological studies using muscle cell impalement have concluded that NO has a functional role in mediating inhibitory neuromuscular relaxation in adult CCM[94-96]. It appears that other putatative neurotransmitters including VIP and ATP have a lesser role and are only likely to act as co-transmitters with NO. The preliminary results of this study are in agreement with these studies[75, 98].

Defective functional nitrergic inhibitory/relaxation has been reported in colonic dysmotility disorders in children[107]. It has been reported that inhibitory/relaxation responses can only be elicited in response to EFS in the ganglionic colonic segments of children with Hirschsprung disease, but not from the aganglionic segments[109]. Limited data is available from studies of adults with acquired megacolon secondary to

idiopathic chronic constipation, where it has been reported that defective nitrergic neuromuscular relaxation might be present[113, 114].

Immunofluorescence staining of nitric oxide nerve density was not available for the specimens used in this study. It will be important to assess whether there is a difference in NO staining density between adult and paediatric STC CCM and to correlate this with a larger series of functional studies. Retrograde labelling and immunofluorescence studies have identified that 45% of motor neurones in adult CCM are inhibitory and that all of these contain nitric oxide synthase[71]. It will be important to determine whether this is also true in paediatric STC.

These results only allow limited conclusions to be made due to the small sample available. The sample size is not sufficient to allow statistical inferences to be made and the results are best interpreted as a pilot study. However, it appears that there might be a difference in the mechanism of inhibitory neuromuscular transmission in adult sigmoid CCM (removed for carcinoma) and transverse CCM from children with slow-transit constipation. Nitric oxide appears to have a predominant role in mediating inhibitory neuromuscular relaxation in adult CCM, but other neurotransmitters are likely to be involved in paediatric STC CCM. Further studies with a larger sample sizes are required to allow interpretation of these results. Firstly, it will need to be determined whether NO also plays a lesser role in mediating inhibitory neuromuscular relaxation in non-STC paediatric CCM. Secondly, immunofluorescence studies are required to assess nerve densities of NO and VIP to correlate with functional studies.

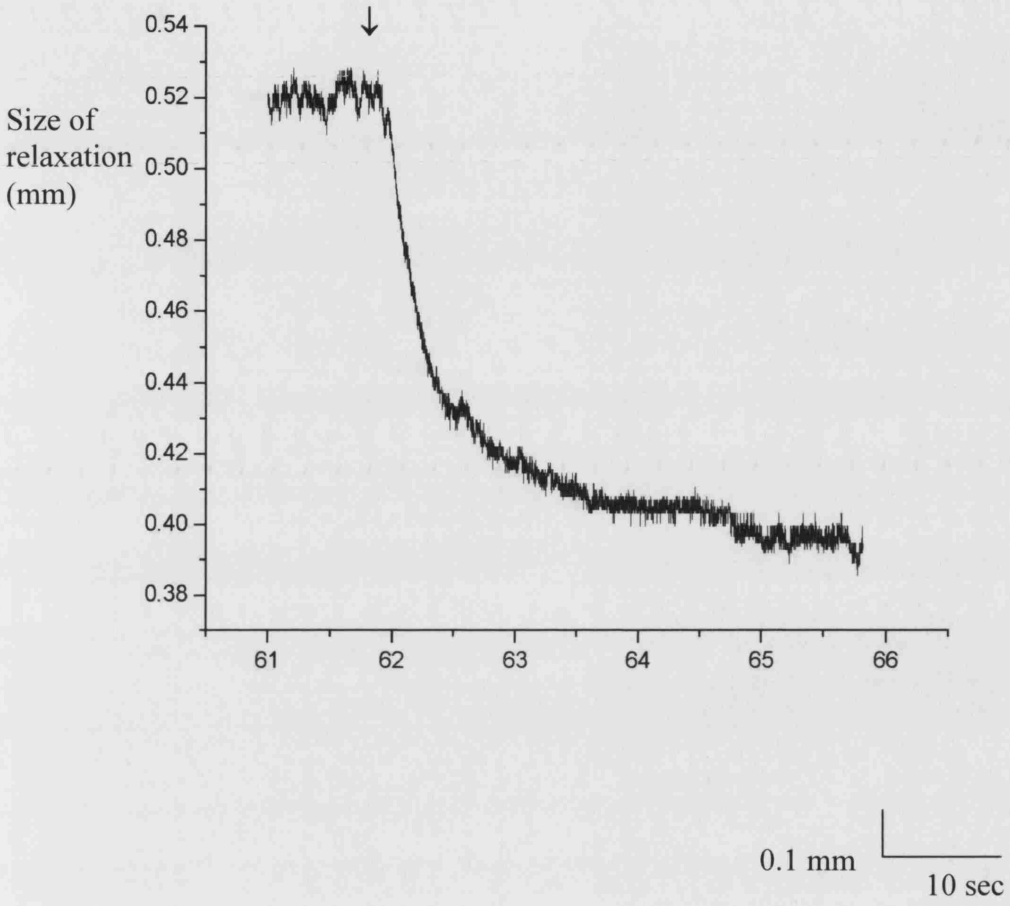

Figure 8: Sodium nitroprusside induces relaxation of colonic circular muscle. Example tracing showing relaxation (mm) induced by 1 μM sodium nitroprusside (SNP) in transverse colonic circular muscle from a child with slow-transit constipation. The arrow indicates the timing of addition of 1 μM SNP to the organ bath.

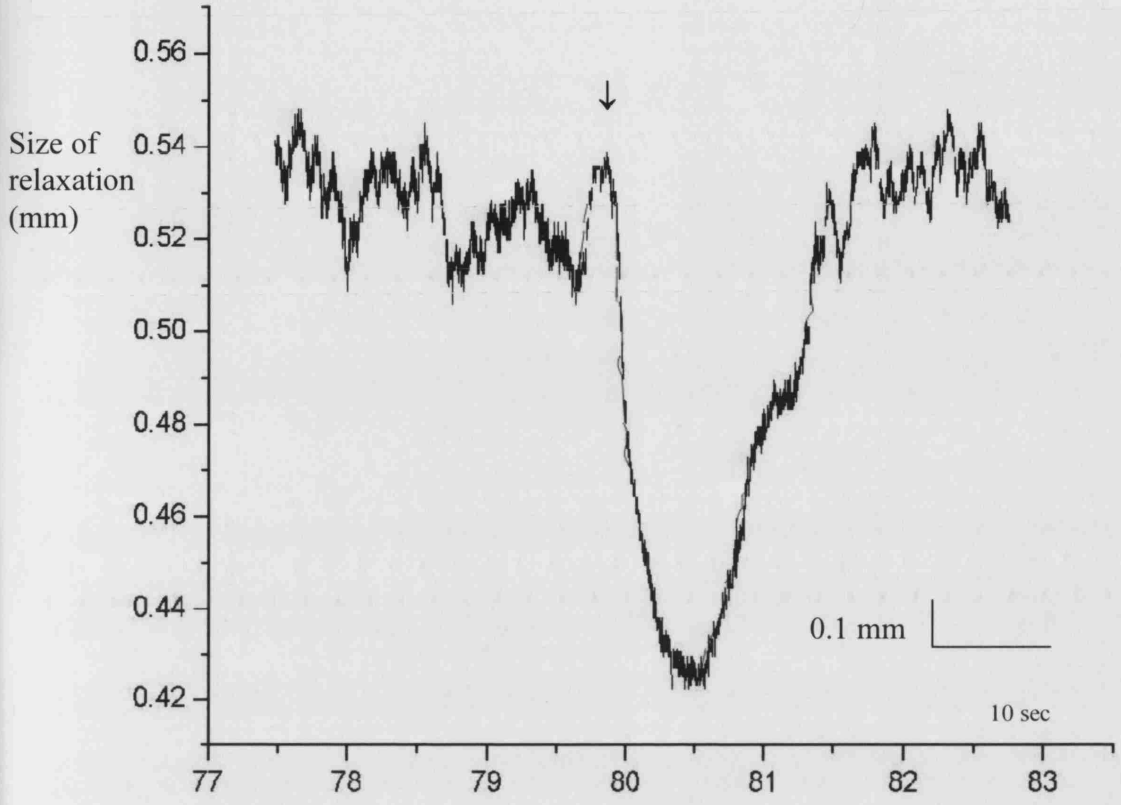

Figure 9: Electrical field stimulation (pulse duration 1.0 msec) elicits relaxation responses in colonic circular muscle. Example tracing showing relaxation induced by EFS in transverse colonic circular muscle from a child with slow-transit constipation. The arrow indicates the start of EFS.

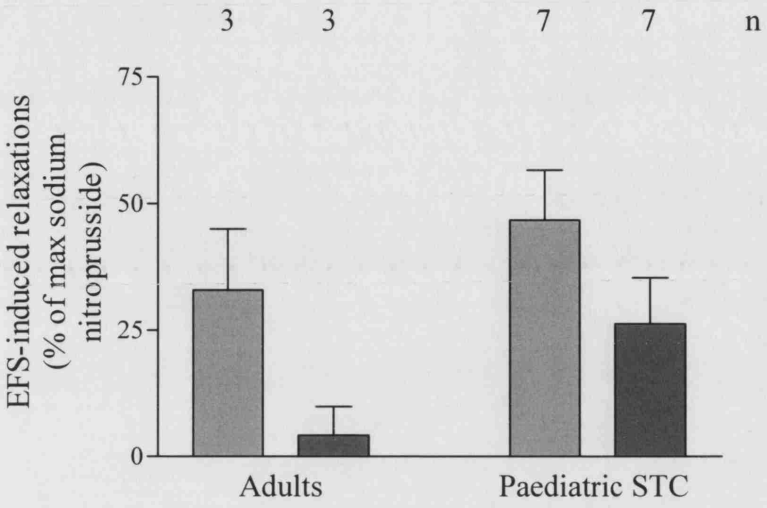

Figure 10: Mean relaxation of colonic circular muscle in response to EFS before and after incubation with 100 μM NOLA. Incubation with 100 μM NOLA (>15 min) reduces relaxations induced by EFS in adult sigmoid and paediatric STC colonic circular muscle. Light bars indicate responses induced by EFS before incubation with NOLA and dark bars indicate responses induced by EFS after >15 min incubation with NOLA. Statistical analyses were not undertaken due to the small sample size.

6. Laparoscopic Placement of Chait Caecostomy Device via Appendicostomy

6.1 Introduction:

We report a technical innovation of inserting the Chait caecostomy device into a laparoscopically formed appendix stoma. Chait *et al*[246] have described the use of a low-profile trapdoor catheter in a percutaneous caecostomy. Our patients and their families report a high degree of satisfaction with the Chait catheter design to, and prefer it to, for example, intubating the stoma with a catheter each time washouts are required. However, we believe that modifying the insertion technique described by Chait *et al* provides several advantages.

This modification creates a mucosa-lined track, rather than the faecal fistula formed by percutaneous caecal placement. The risk of infection is reduced and colonic washouts can be started early. This technique has the further advantage of allowing direct confirmation of position via laparoscopy, avoiding the risk of visceral injury during insertion.

The Malone antegrade continence enema has been successfully used to treat refractory constipation and faecal incontinence secondary to ano-rectal malformations, spina bifida, Hirschsprung disease and slow-colonic transit[38, 268, 269]. Several modifications of Malone's technique have been reported since its first description in 1990[35], including laparoscopic formation[36, 242, 243], in an attempt to improve the outcome of the management of faecal incontinence. The Chait caecostomy technique was first described in 1996[245], however, it is not in widespread use, possibly because of the potential disadvantages of percutaneous placement.

6.2 Methods:

At laparoscopy, the appendix is grasped through a 5 mm trocar placed low in the right iliac fossa and delivered to the exterior. The distal mesentery is diathermied until the tension is released and about 4-5 cm of appendix is below the skin (confirmed laparoscopically). A side-hole is made in the appendix at skin level and the Chait catheter inserted with guidewire assistance and laparoscopic inspection of the appendix (Figure 11 and 12). The distal appendix stump is amputated and the cut end is sutured to the skin using 4 to 8 5/0 absorbable sutures. Washouts are commenced on the first or second post-operative day, as there is no need for adhesions or a track to form.

6.3 Results:

We have undertaken this procedure in 11 children. Laparoscopic appendicostomy with Chait catheter insertion is now the surgical treatment of choice for slow-transit constipation (n = 8) at our institution. We have also carried out this procedure in 3 other patients who had refractory constipation secondary to cystic fibrosis (with distal intestinal obstruction syndrome), sacral agenesis and idiopathic hypotonia. The average age of our 11 patients was 8.7 years, the average length of stay was 4.2 days (to allow parental education) and the mean length of follow-up was 8.1 months. The device was inserted at the primary procedure in 8 out of the 11 patients, and first used for washouts on average 1.6 days post-operatively. Only one of our patients had a minor complication (excess granulation tissue around the stoma, excised as a day case). Otherwise, there were no complications.

6.4 Discussion:

Our series of 11 patients demonstrates that the Chait caecostomy device can be successfully used in the laparoscopically formed appendicostomy. Only one of our patients had a minor complication.

Using the appendix to interpose a mucosa-lined track between the skin and the caecum confers 2 advantages – reduced risk of infection (secondary to faecal reflux) and primary placement of the Chait catheter is possible. Duel and Gonzalez[270] reported superficial infections in 3 out of 5 patients who underwent button caecostomy insertion. A 'small leak' occurred in 1 of the 15 patients first reported by Shandling and Chait[245], this patient required 5 days of intravenous antibiotics. Infections have not occurred in our first 11 patients. We use one dose of peri-operative antibiotic prophylaxis and patients do not undergo pre-operative bowel preparation.

We insert the Chait device at the primary procedure, and so washouts can be commenced on the first 1-2 days post-operatively. Investigators who fashion a caecostomy describe that they do not place their long-term in-dwelling device at the primary procedure, but wait until adhesions and a track have formed and then replace the caecostomy tube at approximately 6-8 weeks post-operatively, to avoid reflux of faeces into the peritoneal cavity[246].

Laparoscopic placement allows direct confirmation of catheter position, and we believe that this minimises the risk of visceral injury associated with 'blind' percutaneous insertion. Several authors have reported that laparoscopic

appendicostomy formation is a safe procedure with low complication rates[243, 244]. One of our previous open appendicostomy cases (a 15 year old male with a previous ano-rectal malformation; not included in the series of 11 laparoscopic insertions) highlights the potential risk of percutaneous insertion. Previously undiagnosed non-rotation of the colon was recognised, prior to attempted catheter insertion. The caecum was located in the left iliac fossa and extensive adhesions to the abdominal wall were present, and so we converted the procedure to open insertion with caecal flap formation. It is probable that significant complications would have resulted if percutaneous insertion had been attempted in this case.

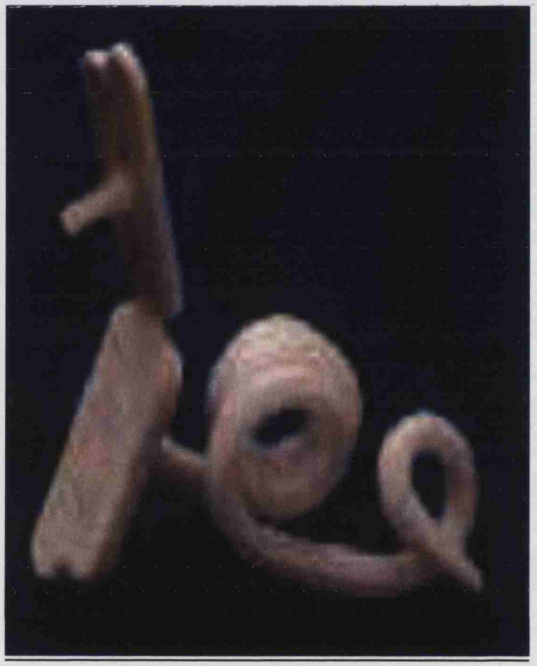

Figure 11: Photograph of a Chait caecostomy device. This shows the helical coils (right side of photograph) that are designed to retain the device within the caecum and the low-profile trapdoor (left side of photograph) through which washouts are delivered.

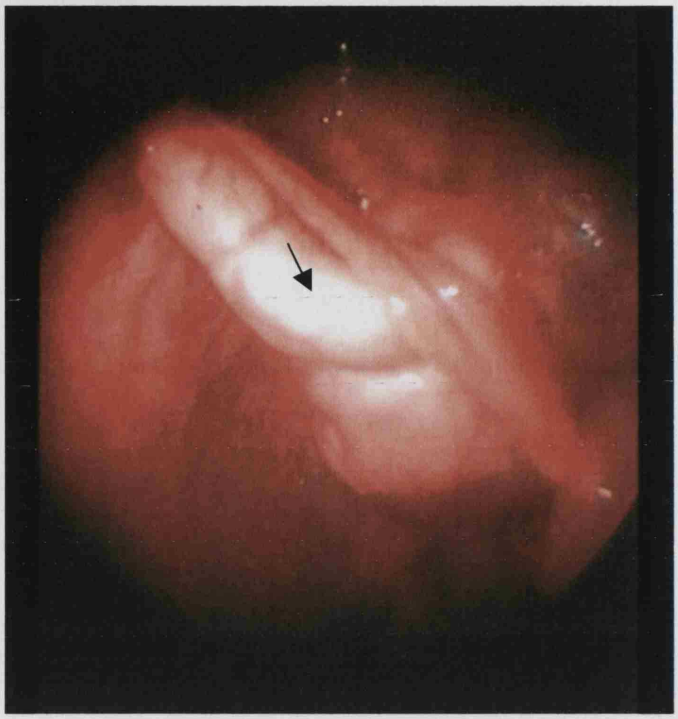

Figure 12: Laparoscopic view confirms position of Chait catheter. The catheter lies within the appendix (arrow); the coils and the tip lie in the caecum.

7. Colonic manometry shows reduced frequency, amplitude and length of propagating sequences in children with slow-transit constipation.

7.1 Introduction:

Chronic constipation is common in children and most respond to simple treatments such as behavioural modification and laxatives[5]. Some are resistant to standard therapies and may present with disabling soiling[5]. Two major pathophysiologies have been identified in chronic constipation: slow transit constipation (STC) or colonic inertia, and pelvic floor dysfunction leading to anorectal retention[271]. It has been suggested, however, that slowed colonic transit in childhood might be an epiphenomenon to the constipation itself[13] or secondary to behavioural disturbances and toilet refusal[129]. Certainly, the pathological basis of slow colonic transit in childhood is poorly understood.

Movement, mixing and storage of colonic contents in healthy adults involves contractions that are both propagating and non-propagating[42]. About 1/3 of antegrade low-amplitude propagating sequences (PS) (amplitude < 90 mmHg) are associated with antegrade movement of colonic content[42]. High-amplitude PS (HAPS) are thought to represent 'mass movements' of colonic contents which can also be observed radiologically[219]. Chronic constipation in adults is associated with a reduced frequency and amplitude of HAPS[46, 224]. The response to a meal is also abnormal, with no post-prandial increase in colonic motor activity, and a failure to move intraluminal contents[213]. There are considerably fewer colonic manometric

studies available from children with otherwise uncomplicated chronic constipation[43] and none in children with established slow transit constipation.

Manometric recordings from the colon of children to date have only been achieved using endoscopically placed catheters passed "retrogradely", usually as far as the transverse colon[44]. Endoscopic placement introduces the risk of procedural morbidity, the right side of the colon is not included in the study and the catheter may easily become displaced. Per-nasal passage of a trans-intestinal naso-colonic catheter has proven an effective means of studying colonic motility in adults[1], however it can be very difficult to obtain consent for pernasal intubation of the gastrointestinal tract in children and adolescents. There are no published studies using this type of manometric instrumentation in children.

There is increasing acceptance and use of appendicostomies as a mechanism for colonic irrigation and controlling faecal retention in children with otherwise uncontrollable faecal soiling and constipation[38, 272]. We recognized that this could provide a route to assess pan-colonic motor activity for prolonged periods in a previously little-studied group. The aim of our study was to develop a technique for the study of colonic motility by placing the colonic manometric catheter through an established appendiceal stoma. We hypothesized that children with slow transit constipation would have a reduction in the strength, coordination and propagation of colonic contractions.

7.2 Methods:

7.2.1 Patient Selection:

We contacted the parents of 40 sequential children who had undergone appendicostomy formation for treatment-resistant constipation and soiling with slow transit constipation through the Department of General Surgery at the Royal Children's Hospital, Melbourne. All had been operated upon at least 6 months previously. Six children and their parents agreed to participate in the study. Their mean age was 11.5 years (range 8-16 years) and five of the six were boys. Slow colonic transit was defined on scintigraphic transit study as retention of radio-isotope in the ascending, transverse and descending colon beyond 48 hours[273]. Hirschsprung disease had been excluded by rectal biopsy. Informed written consent was obtained from the parents (and child where applicable). The study protocol was approved by the Ethics in Human Research Committee at the Royal Children's Hospital, Melbourne (Reference Number 21049 A).

7.2.2 Manometry Assembly and Catheter Design:

The manometry catheter was a custom-designed, balloon tipped, 9 lumen (8 channel) extruded silastic catheter, with an external diameter of 3.5 mm (Dentsleeve, Adelaide, South Australia). The catheter intersidehole distance was 7.5 cm, and the total length of the catheter was 180 cm. The central lumen inflated a 5 ml Foley-type balloon positioned 2 cm proximal to the tip of the catheter. The centre of the catheter was coated with barium sulphate, to facilitate fluoroscopic visualisation. We used a Neomedix pneumohydraulic perfusion assembly (Neomedix Systems Pty Ltd, Warriewood, New South Wales, Australia) to pump distilled water through the

catheter lumens at a total catheter perfusion rate of 0.25 mL/min. The recording lumens were connected to external pressure transducers (Neomedix Systems Pty Ltd). Data were amplified, digitised and recorded at 10 samples/second using GastroMac version 3.2 Aquidata software (AD Instruments Pty Ltd/ Neomedix Systems Pty Ltd).

7.2.3 Study Protocol:

All six patients had been performing regular antegrade colonic saline washouts 2-3 times weekly for at least 6 months prior to the study. Thus, all of the children were studied in the 'prepared' state, and did not have colonic distension or an obstructing faecaloma. Irritant laxatives were not being used and any other aperients (if used) were ceased at least 5 days prior to the start of each study. Patients underwent per-appendicostomy manometry catheter placement, having carried out their usual colonic washout on the preceding day. Patients were allowed to eat and drink normally on the day of catheter insertion. Local anaesthetic (1% lignocaine gel) was applied to the stoma and peri-stomal skin, and any in-dwelling appendicostomy device (e.g. Chait caecostomy button[246] was removed. A 10 Fr feeding tube was then passed via the appendix stoma and bisacodyl solution (2-4 mg) was instilled directly into the caecum. Bisacodyl is a stimulatory and osmotic laxative and was used to aid propulsion of the catheter.

The manometry catheter was inserted into the caecum and advanced in an antegrade direction along the colon. The balloon at the tip of the catheter was inflated with 3 mL of water to aid propulsion. Catheter advancement took between 10 minutes and 4

hours, during which time the patients were fully ambulant and ate or drank normally. Fluoroscopic visualisation (< 30 sec per patient) was used to check the position of the catheter. Once the catheter tip reached the recto-sigmoid region, and all of the sideholes were located within the colon, the external portion of the catheter was fixed in position to the skin of the patient's abdomen with adhesive tape (Figure 13). Neither sedation nor analgesia was used for any patient. Patients were allowed to return home for overnight stay on day 1 if they wished.

The following day, the position of the catheter was confirmed by fluoroscopy prior to starting recordings. The manometry catheter was then connected to the recording assembly via external pressure transducers. Each study began at approximately 10 am (i.e. more than 24 hours after bisacodyl instillation and catheter insertion, in order to avoid any residual effect of bisacodyl) and recordings continued for 20-24 hours, during which time the child/parents kept an event diary. This documented activities such as eating, changes in posture, sleeping, micturition, abdominal sensations, passage of flatus and spontaneous defecation. The subject remained recumbent or semi-recumbent for the 24-hour period of each study.

A standardised dietary intake was eaten by each child during the manometric recording. This consisted of 45% fat, 34% carbohydrate, 17% protein. The calorific content of the breakfast was 300 kCal; lunch and dinner were both 1000 kCal.

On completion of each recording, the catheter position was again confirmed fluoroscopically. The balloon was then deflated, and the catheter withdrawn and removed through the appendix stoma.

7.2.4 Data Analysis:

As this study examined children who had previously undergone appendicostomy formation, it was not possible for ethical reasons to study healthy children using a similar method of catheter insertion. Therefore, we compared results to data collected by Bampton *et al*[1] in their study of 14 healthy young adults (mean age 25 years) using nasocolonic intubation. We matched the experimental conditions used by Bampton *et al*[1] by using a similar catheter design, dietary intake and data analysis parameters. Thus, antegrade or retrograde propagating sequences (PS) were defined as monophasic pressure waves of amplitude > 5 mmHg, occurring in 3 or more adjacent sideholes, with an inter-sidehole velocity of 0.2-12 cm/sec. Propagating sequences were classified as high amplitude propagating sequences (HAPS) if the amplitude of at least one component was greater than 116 mmHg (representing the mean amplitude of colonic propagating pressure waves at mid colon, plus two standard deviations[1]). In our study, we identified candidate PS and HAPS by reviewing the 24-hour trace visually, and used computer assistance to measure the amplitude and inter-sidehole velocity of each. Area-under-the-curve analysis was performed to calculate non-propagating colonic activity for 120-minute epochs both before and after the standard lunch. For these time epochs, the baseline of activity was re-set to 0 mmHg in each channel for that epoch. Artefactual activity, for example cough or micturition strain, was removed from the analysis and replaced with mean area under the curve values

for the same time period. Pre- and post-prandial non-propagating activities were compared, as were changes after wakening.

For antegrade, retrograde and high-amplitude propagating sequences, the frequency, amplitude and distance of propagation in patients were compared to controls using unpaired t tests. Colonic regions were identified based on sidehole positions: caecum, ascending colon, hepatic flexure, mid-transverse colon, splenic flexure, descending colon, sigmoid and rectosigmoid. Regional variations in the frequency, amplitude and velocity of PS within colonic regions were assessed using ANOVA for repeated measures. The mean area-under-the-pressure-curve in the time epochs before and after meal ingestion and waking was compared using paired t tests. The frequency of events was expressed in terms of 24-hour periods. All data are expressed as mean ± SEM, unless indicated otherwise.

7.3 Results:

7.3.1 Catheter Placement and Recording Times:

The catheter tip was located in the distal sigmoid colon in 5/6 patients (Figure 13). In one (#2) the catheter only progressed as far as the splenic flexure. No child experienced significant discomfort or requested analgesia during or after catheter insertion or removal. No complications were associated with these procedures.

7.3.2 Frequency of Propagating Sequences (PS) in STC (Table 4):

Antegrade, retrograde and HAPS were less frequent in STC children (Table 4). One patient had little activity with no antegrade or retrograde PS and only 3 HAPS in 24 hrs. Four patients had few antegrade PS and only one patient had similar numbers of antegrade PS as healthy young adults[1]. The mean frequency of antegrade PS in 6 STC patients was significantly less than in healthy young adults (STC 13 ± 6 /24 hrs, controls 52 ± 6 /24 hrs; $p < 0.01$). In the STC patients, there was no significant variation in PS frequency in relation to colonic region. In healthy young adults, the PS frequency was highest in the caecum and decreased distally (ANOVA; $p < 0.0005$)[1].

The right colon is a major site for mixing of stool. This is achieved by both antegrade and retrograde PS. Retrograde PS were absent from 3/6 STC patients, the mean frequency was 10 ± 7 /24 hrs which was less than but not significantly different to normal young adults (17 ± 5 /24 hrs; $p = 0.44$). The mean number of antegrade and retrograde PS was similar in STC patients. In contrast, control subjects had 3 fold more antegrade than retrograde PS ($p < 0.001$).

HAPS are thought to produce mass movements. HAPS were not present in 2/6 patients, and in the other 4 the frequency of HAPS was low compared to healthy young adults (STC 5 ± 2 /24 hrs, controls 9.9 ± 1.4 /24 hrs; $p < 0.05$). In one patient a retrograde HAPS was recorded; these were not observed in controls.

7.3.3 Amplitude of Propagating Sequences in STC (Table 4):

The mean amplitude of antegrade PS (39 ± 9 mmHg) was 27% less than controls (54 ± 3 mmHg; $p < 0.05$), while the mean amplitude of retrograde PS (43 ± 6 mmHg) was 37% higher than controls (27 ± 1 mmHg; $p < 0.01$). There was no difference in the amplitude of antegrade PS as they propagated in STC colon compared to controls ($p = 0.97$). Antegrade pressure waves were significantly greater in amplitude in the descending colon of healthy young adults ($p < 0.03$). In STC patients, the mean amplitude of component pressure waves in HAPS (94 ± 10 mmHg) was significantly less than healthy adults (117 ± 3 mmHg; $p < 0.01$). There was no change in the mean amplitude of HAPS with distance propagated in STC patients ($p = 0.49$).

7.3.4 Site of Origin of Propagating Sequences:

The most common site of origin of antegrade PS was the caecum (7.2 ± 4.0/24 hr). This decreased in more distal regions but the difference was not statistically significant ($p = 0.58$). In controls, antegrade PS (7.7 ±1.2 /24hr)[1] and HAPS originated more frequently in the caecum than other regions ($p < 0.0005$). In STC patients HAPS originated most frequently in the descending colon (3.5 ±1.5 /24hr; $p < 0.05$), (Figure 14).

7.3.5 Propagation Distance and Velocity:

The distance propagated (number of sideholes) by antegrade or retrograde PS was similar for STC patients and healthy young adults (Table 4), but there was a significantly shorter distance propagated by HAPS in STC patients ($p < 0.05$).

Antegrade PS and HAPS had similar velocities in STC, while HAPS were significantly slower than antegrade PS in healthy young adults. There was no significant difference in the velocity of antegrade PS, retrograde PS or HAPS in STC in different regions (Figure 15). In healthy young adults the velocity of antegrade and retrograde PS increased significantly as the waves propagated[1].

7.3.6 Colonic Meal and Waking Responses:

Unlike controls, STC patients did not show an increase in non-propagating colonic activity in response a 1000 Kcal meal (1-0 hr pre-prandial motility index 160,029 ± 19,313 mmHg.sec compared to 0-1 hr post-prandial motility index 167,013 ± 11,412; $p = 0.73$). Controls demonstrated a post-prandial increase in HAPS ($p < 0.03$), but this was seen in only one of six STC patients ($p < 0.001$). Following morning wakening, controls had a significant increase in non-propagating activity ($p < 0.0001$); there was no significant change in motility index in STC patients (1-0 hr pre-wakening 161,587 ± 50,081 mmHg.sec compared to 0-1 hr post-wakening 179,510 ± 11,091; $p = 0.74$).

7.4 Discussion:

To our knowledge, this is the first study to achieve pan-colonic manometric recordings in children with well-defined slow transit constipation. Where previous paediatric studies of colonic motor function have been carried out, the type of constipation has generally been poorly-defined, with failure to distinguish between slow transit constipation and pelvic floor dysfunction[43, 44]. Past studies in children have also been of short duration (4 hours) and employed catheters inserted during colonoscopy[43, 44]. Insertion during colonoscopy is usually performed under sedation

and has been used to perform colonic manometry in both adults[79, 213, 218, 219] and children[43, 44, 227, 228] with colonic motility disorders. A significant limitation of colonoscopic insertion with a guidewire is that the manometry catheter is usually not passed to the right side of the colon and so studies are in effect limited to motor events from transverse colon to rectum. The catheter itself may easily become displaced. Our technique was able to measure changes throughout the entire colon. Rao *et al*[214] have reported that colonoscopic placement of motility catheters can be performed in adult volunteers without sedation, but this would contravene currently accepted safe practice for colonoscopy in children[274]. Colonic instrumentation, sedation and the presence of a catheter passing per-rectum may interfere with colonic motility. Some colonic motor events such as HAPS are infrequent and abbreviated 4-hour recordings may miss them completely.

Per-nasal placement of a colonic catheter has been developed[1, 216, 218, 219]. This technique is feasible in adults but repeated per-nasal catheter advancements are necessary and per-nasal catheterisation can be difficult for children to tolerate[275]. The present study overcame problems associated with colonoscopic or per-nasal catheter insertion by placement of the catheter through an existing appendix stoma. This was well tolerated by children, enabling prolonged studies of colonic motor activity.

All 6 patients with slow colonic transit had abnormal colonic pressures and motility. It has been suggested that chronic constipation in children has a psychological basis[43]. Our studies identify specific physiological abnormalities. The colons of STC children

had reduced activity and ability to propagate contractions compared to healthy young adults. We found that propagating sequences in the colon of children with STC were markedly reduced in frequency, amplitude and distance of propagation. In addition, children with slow colonic transit did not have an increase in colonic activity following waking or eating a meal. The presence of HAPS and post-prandial increase in colonic activity is characteristic of normal colonic motility[1]. Di Lorenzo *et al*[43] studied transverse and descending colonic motility in children over a 4-hour period using an endoscopically-placed catheter. They found that children with functional faecal retention had an increase in post-prandial motility index and at least one HAPS as compared to patients with a neuropathy in whom there was neither a post-prandial change nor any HAPS. Patients with hollow visceral myopathy had no demonstrable contractile activity. The authors did not determine whether their patients with functional faecal retention had either slow transit constipation or pelvic floor dysfunction. In a further study using the same methodology[44], children with a probable neuropathic form of chronic intestinal pseudo-obstruction had no post-prandial increase in motility index and 12 of 16 had no demonstrable HAPS. Colonic transit times were again not defined. In contrast, children with repaired ano-rectal malformations have excessive numbers of HAPS into the neorectum as well as internal anal sphincter dysfunction[227]. Given the relative infrequency of HAPS in normal adults (approximately 10 in each 24 hours)[1], 4-hour studies are unlikely to adequately reflect reduced HAPS activity. Studies by Bampton *et al*[1] and others have also established important regional differences in colonic motility in normal young adults. The caecum was the most frequent site of origin of antegrade PS and HAPS in normal subjects. The amplitude of antegrade PS increased as they travelled from the right to the left side of the colon. In our studies of children with STC, these regional

variations were absent. Regional and diurnal variations in colonic activity, and responses to meal ingestion, underline the importance of prolonged manometric studies in understanding the pathophysiology of colonic activity[219]. This lack of regional variation in colonic motility may well be of importance in slowing colonic transit in STC.

In this study, we found that retrograde PS occurring in the colons of these STC children had greater amplitude than retrograde PS in healthy young adults. Together with a decrease in antegrade PS, this suggests there is an imbalance in the antegrade and retrograde movement of colonic content in children with slow colonic transit. The contractile properties of the 6 patients varied, suggesting they still represent a heterogeneous group, perhaps with different defects underlying the impaired motility in each (this also implies that there was minimal, if any, effect from the administered intra-caecal bisacodyl used during catheter insertion). A change in the balance of excitatory and inhibitory transmission could affect the overall strength of contraction, or change the coordination of relaxation and contraction. Various neuronal defects have been described with slow transit constipation, including reduced substance P and enkephalin in excitatory nerve fibres in the colonic circular muscle[31, 262], VIP in inhibitory nerve fibres[112, 114, 276], neuroendocrine peptides- enteroglucagon and serotonin in mucosal cells[277, 278], decreased interstitial cells of Cajal[53] and increases in calcitonin gene related peptide[276]. All of the children we studied had reduced substance P in colonic circular muscle, but future histochemical studies will be need to define the range of defects in the enteric nervous system that result in slow transit through the colon.

The natural history of childhood slow transit constipation is not known. It has previously been unclear whether adult slow transit constipation first develops in adult life, or has its origins in childhood. There is a well-recognized subset that presents after childbirth. Manometric studies of contractile activity in adult slow transit constipation have shown similar findings to ours, with a reduction in the frequency, amplitude and duration of propulsive HAPS[225]. Long-term follow-up of patients presenting with STC in childhood will be needed in order to understand its progression though adult life.

We and other authors[1, 43, 44, 46, 225] define very different pathophysiological events in slow transit constipation as compared to pelvic floor dysfunction. It is possible that a better understanding of these factors may lead to more rational therapies, which at present rely on behavioural modification techniques and simple laxatives[5]. As a technique, colonic manometry may well find other therapeutic uses. Martin *et al*[232] and Di Lorenzo *et al*[228] have reported recently on the use of colonic manometry in Hirschsprung disease as a pre- and post-operative guide to surgical management, such as the timing of stoma formation and stoma closure.

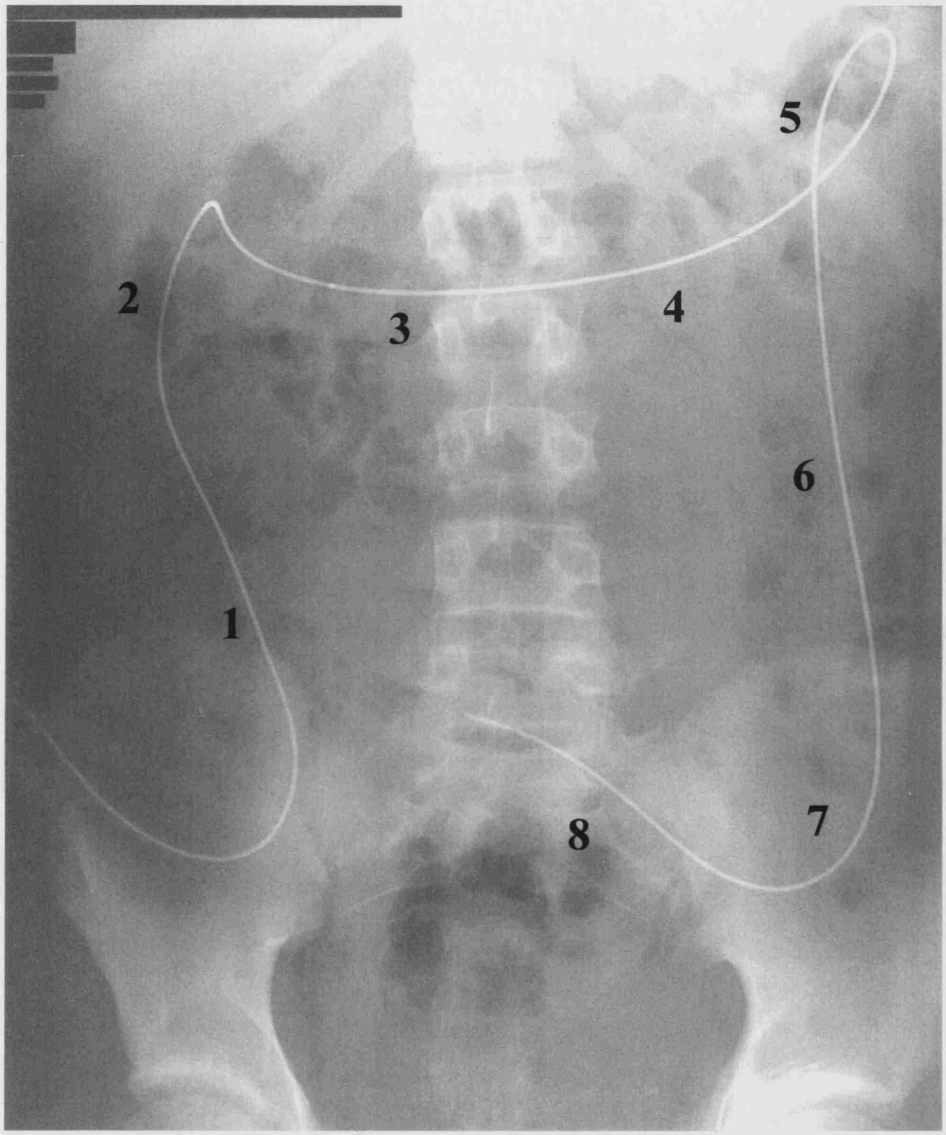

Figure 13: Abdominal X-ray showing position of 8 sidehole colonic manometry catheter. The catheter has been inserted via the appendicostomy and the tip is positioned in the distal sigmoid colon. Numbers 1-8 indicate the position of the sideholes, spaced at 7.5 cm intervals from the appendix (1) to the distal sigmoid (8).

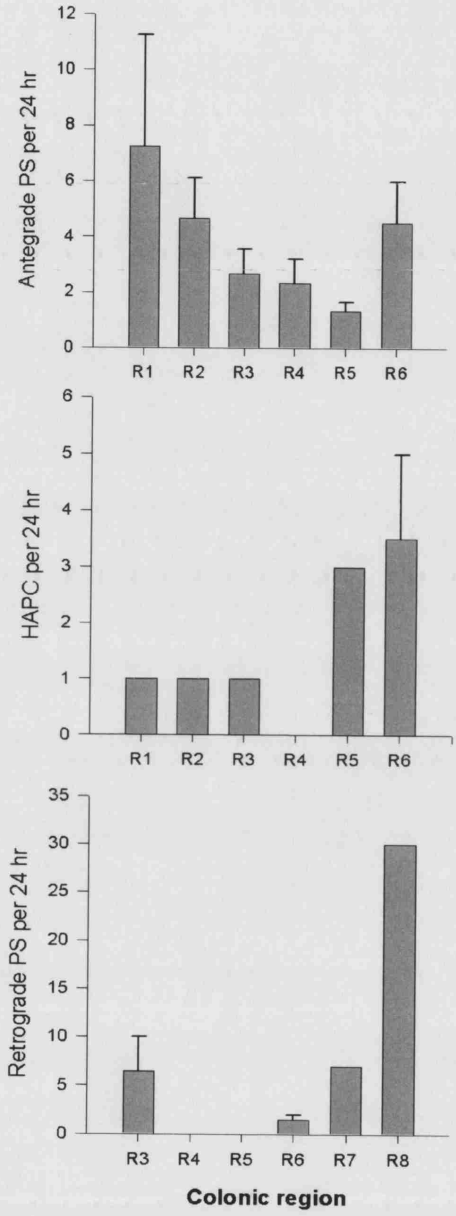

Figure 14: Site of origin of antegrade propagating sequences and HAPS. Colonic regions of origin R1 to R6 correspond to caecum, ascending colon, hepatic flexure, mid-transverse colon, splenic flexure and descending colon respectively. Data are given as mean ± SEM. * $p<0.05$ (ANOVA).

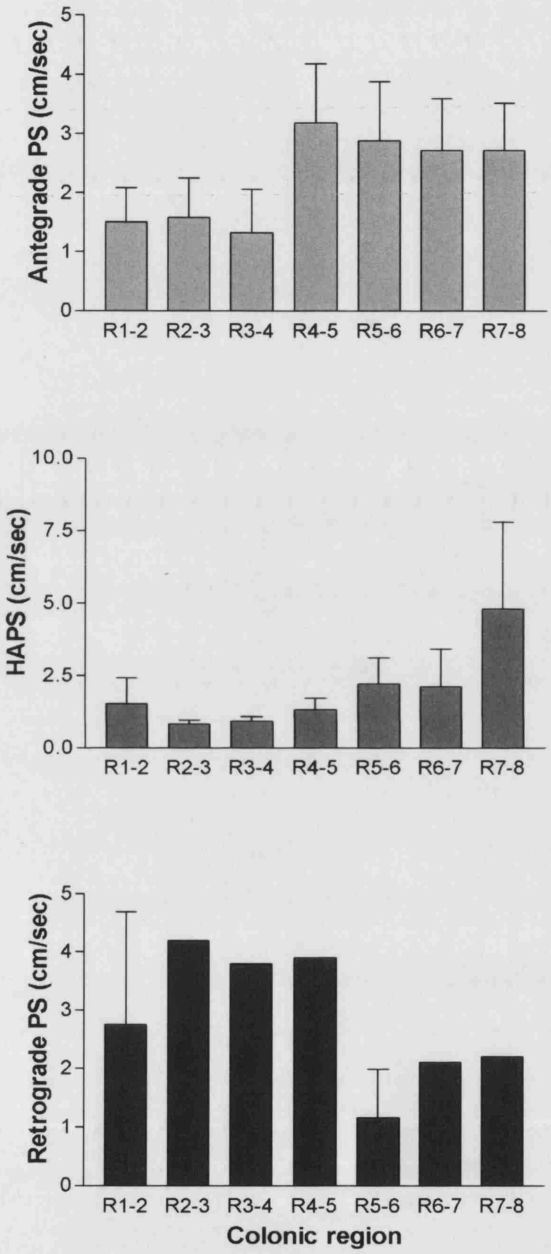

Figure 15: Velocity of antegrade PS, HAPS and retrograde PS. Colonic regions of origin R1 to R6 correspond to caecum, ascending colon, hepatic flexure, mid-transverse colon, splenic flexure and descending colon respectively. Data are given as mean ± SEM.

Patient	Diagnosis	Age (yrs)	Duration (hr. min)	Frequency (/24 hr)			Amplitude (mmHg)			Distance (SH)			Increase in Response to meal	
				APS	RPS	HAPS	APS	RPS	HAPS	APS	RPS	HAPS	AUC	HAPS
1	STC	16	23.3	42	39	0	36	35	0	5.2	4	-	N	N
2	STC	11	20.0	0	0	3	0	0	82	-	-	6.4	N	N
3	STC	11	23.0	14	0	14	53	0	77	5	-	4.7	Y	Y
4	STC	14	23.4	7	18	0	26	54	0	3	3.2	-	N	N
5	STC	8	23.5	6	0	4	15	0	94	4.2	-	4.5	N	N
6	STC	9	21.0	8	2	7	67	41	124	3.9	3	3.5	Y	N
STC Patients		11.5 ± 1.2		13 ± 6** (0-41)	10 ± 7 (0-38)	5 ± 2* (0-13)	39 ± 9*	43 ± 6**	94 ± 10**	4.3 ± 0.4	3.4 ± 0.3	4.8 ± 0.6*	2/6	1/6
Adult Controls		25 ± 0.75	24.0	52 ± 6 (28-115)	17 ± 5 (1-70)	9.9 ± 1.4 (3-24)	54 ± 3	27 ± 1	117 ± 3	4.8 ± 0.2	3.2 ± 0.1	6.2 ± 0.3	25/25	25/25

Table 4: Age, frequency, amplitude and distance travelled by propagating sequences (PS) and meal response (area-under-pressure-curve and HAPS frequency) in 6 children with STC and 14 healthy adults. Recordings derived from 5 channels from patient 4. Data presented as mean ± SEM with range in brackets. APS – antegrade

propagating sequences, RPS – retrograde propagating sequences, HAPS – high-amplitude propagating sequences, SH – sideholes (inter-sidehole distance 7.5 cm), AUC – area-under-the-curve indicates increase in colonic activity. Meal response indicates significant increase in motility index during the hour before and the hour after a meal or increase in HAPS; Y = yes, N = no increase. Adult control data derived from Bampton *et al*[1]. ** $p < 0.01$ * $p < 0.05$.

8. Overall conclusions:

Colonic dysmotility in children remains a significant clinical problem. A minority of children with constipation develop chronic, intractable constipation and soiling[9], but most do not have colonic aganglionosis (Hirschsprung disease). In clinical practice, such children are usually labelled as having 'idiopathic constipation' and are managed with empirical medical treatments such as laxatives and enemas. A range of diagnoses has been proposed to classify this heterogeneous group of children, including intestinal neuronal dysplasia, hypoganglionosis and chronic intestinal pseudo-obstruction. These disorders have controversial diagnostic criteria and this underlines the fact that further research is required in terms of aetiology, diagnostic criteria, clinical investigations and both medical and surgical treatment modalities[148, 153, 179]. The need for research into the areas of functional constipation and neuronal dysplasias in children is highlighted in the recommendations of the Research Agenda for Paediatric Gastroenterology in a report published in 2002 by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition[9]. The overall hypothesis of this thesis is that children with chronic idiopathic constipation may have symptoms attributable to an intrinsic colonic motility defect, rather than primary behavioural or psychological problems. The objective of this series of studies was to determine if this group of children have demonstrable defects in colonic physiology, firstly by sub-classifying them using radiological methods and then by applying *in vitro* and *in vivo* techniques to measure colonic motor characteristics.

The meta-analysis of 9 studies reporting gastro-intestinal and/or colonic transit times in healthy children in this thesis has demonstrated that the normal mean colonic transit

time in children is 30 hours and the upper limit of normal transit of 58 hours. This provides a basis to define slow-transit constipation in children radiologically. Scintigraphy appears to have an established role in the investigation of adults with chronic intractable constipation, but is not in widespread use in the investigation of children with colonic motility disorders.

In this study, the reported advantages of scintigraphy over radio-opaque marker studies in the assessment of adults with chronic constipation were shown to apply to the investigation of children. Multiple images can be acquired for 48 hours following ingestion of the radio-isotope without increasing the radiation dose received by the patient. This allows the assessment of gastric and small bowel motility, allowing the identification of children with pan-intestinal motility disorders.

By acquiring images at 0-2 hours, 6 hours, 24 hours, 30 hours and 48 hours, three patterns of colonic transit were identified – slow colonic transit, normal colonic transit and functional faecal retention (obstructed defaecation). This differentiation requires a combination of visual assessment of scintigraphic images and calculation of the geometric centre of radioactivity at each imaging time. The geometric centre of radioactivity was found to be insufficiently accurate to permit categorisation of colonic motility patterns. As the upper limit of colonic transit in the meta analysis of studies reported on healthy children was 58 hours, it is likely that a further imaging episode at 56-60 hours post-isotope ingestion would be beneficial in determining the 3 patterns of transit. It is important to distinguish slow colonic transit from normal colonic transit and functional faecal retention, as these patients with slow-transit constipation require different treatment strategies.

Having established a basis for the diagnosis of slow-transit constipation in children, it has been possible to proceed to investigate possible neuronal or smooth muscle anomalies in the colon of children with this disorder. *In vitro* studies of colonic circular muscle biopsies obtained at laparoscopy were carried out. It has been reported previously that tachykinin immunoreactivity is reduced in CCM of adults[31] and some children[6, 172] with slow-transit constipation. Currently, no studies have been carried out to describe the staining densities of other enteric neurotransmitters such as acetylcholine, nitric oxide and vasoactive intestinal peptide in the colon of children with slow-transit constipation. The potential significance of the histological finding of low TK-IR can only be fully evaluated by correlation with functional studies. This is the first study to attempt to define functional neuromuscular transmission in children with STC.

It can be concluded from the *in vitro* contractility studies undertaken that there is no defect in cholinergic neuromuscular transmission in the transverse colonic circular muscle in children with STC. This contrasts with reports on colon from adults with slow-transit constipation. Cholinergic neuromuscular transmission is unaffected in children with STC, whether tachykinin immunoreactivity in the CCM is normal or low.

Tachykinins appear to have a role in neuromuscular transmission in adult transverse CCM, in agreement with other reports[86, 88, 90]. However, no role was found for tachykinins in neuromuscular transmission in transverse CCM from children with STC, whether TK-IR was normal or low. NK_2 receptors do not appear to be defective

in this disorder, as addition of the natural endogenous ligand (NKA) elicited contractions in all specimens. It is possible that NKA synthesis or release may be defective.

An interesting issue raised by these studies is the residual contraction seen in CCM from both the adults and the children with STC in the presence of full cholinergic and tachykininergic blockade. This contraction was not due to direct electrical stimulation of the muscle strips as further reduction or abolition of contraction was noted after addition of the neurotoxin tetrodotoxin. This implies that there may be neurotransmitters, which mediate excitation/contraction in the human colon that is not cholinergic or tachykininergic in nature. The possibility that this mechanism could be defective in children with STC may be an area for further research.

It would be ideal to compare the findings in CCM from children with STC to age-matched children with no gastro-intestinal symptoms, due to possible age-related changes in neuronal numbers in the myenteric plexus[279]. However, this is clearly not possible for ethical reasons. Therefore, the best available alternative, i.e. adult colon taken from specimens resected for carcinoma, was used.

The aetiology of slow-transit constipation in children is likely to prove to be multifactorial and the complexity of the enteric nervous system means that a single neurotransmitter defect may not necessarily result in an easily identifiable functional deficit. For example, NK_1 receptors are found at multiple locations[82, 83, 264] and so a reduced nerve fibre staining density may produce a combined alteration in excitation, inhibition and interneuronal modulation. However, it may be that multiple

nerve fibre density abnormalities may be determined in the CCM of children with STC and this may form the basis of a classification system. Further research using immunofluorescence staining is required to ascertain whether there are abnormalities in the neurotransmitters acetylcholine, nitric oxide and VIP.

The preliminary results of the CCM relaxation studies reported in this series suggest that there may be a difference in nitric oxide mediated inhibition/relaxation between adult colon and colon from children with STC. This study could be expanded in two ways. Firstly, the same protocol could be used with a greater sample size. Secondly, agonists and antagonists to other proposed mediators of inhibition/relaxation, such as vasoactive intestinal peptide, could be tested to address this issue.

It has been reported that the ICCs have a role both as pacemaker cells and as modulatory cells[50, 51, 116]. Immunofluorescence staining techniques are available to determine levels of interstitial cells of Cajal (ICCs). Antibodies for the *c-kit* receptor, which is located on ICCs, has allowed assessment of densities of these cells in the gut. Abnormalities in ICC levels have been reported in association with Hirschsprung disease and chronic idiopathic pseudo-obstruction syndrome[52, 152, 280]. Studies of ICC structure and function in children with STC are required.

Appendicostomy formation is a widely used surgical intervention to allow access via the appendix into the caecum and the proximal colon in order to carry out antegrade colonic washouts[35, 37, 268]. The modification of the appendicostomy technique described here involves using laparoscopy to create the stoma and the use of a low-profile trapdoor appendicostomy device that can be inserted at the primary procedure.

These modifications avoid the potential risks of percutaneous caecostomy formation. A mucosal conduit (the appendix) is used between the skin and the colon, rather than a faecal fistula established by percutaneous insertion and so the risk of infection is reduced. Direct visualisation of the appendix and subsequent insertion of the Chait device into the caecum using laparoscopy reduces the risk of visceral injury that may be associated with 'blind' percutaneous insertion. This series of 10 patients demonstrates that this minimally invasive technique is safe and allows washouts to be commenced early in the post-operative period.

Colonic manometry is a difficult procedure to undertake in children, due to the problems of accessing the colon. However, there is great potential for gaining insights into *in vivo* colonic motility patterns. Previous reports of paediatric colonic manometry have described catheter insertion using colonoscopy with sedation/anaesthesia[41, 43, 44]. In order to overcome these problems, we proposed a technique for colonic catheter intubation that can be carried out without sedation. Per-appendicostomy placement of a manometry catheter into the colon has not been reported previously in children. This was tolerated well by the 6 children studied in this report, all of whom had scintigraphic STC. Twenty-four hour colonic manometry in these children was possible and recordings of propagating and non-propagating colonic motor activity, including responses to meal ingestion and waking were undertaken. Data obtained was compared to that recorded from 14 healthy young adults using similar catheter design, experimental protocol and data analysis.

The results of colonic manometry in children with STC indicated that there is a physical basis to the delayed transit seen on scintigraphy that can be demonstrated *in*

vivo. Children with STC displayed a reduced frequency of antegrade and high-amplitude propagating sequences, a reduced amplitude of high-amplitude propagating sequences and a reduced distance of propagation of high-amplitude propagating sequences. The children studied did not demonstrate the normal increase in non-propagating colonic activity in response to eating a meal seen in healthy adults.

The successful use of the appendix stoma as a conduit to undertake prolonged colonic manometry studies has opened up further possibilities for the investigation of colonic motility disorders in children. The patients assessed in this study all showed abnormal 24-hour colonic manometric profiles, when compared to adult values. However, even within this relatively small population, differences in these abnormalities are apparent in terms of the numbers of antegrade, retrograde and high-amplitude propagating sequences. By studying a larger group of children it may be possible to sub-classify children with radiological slow colonic transit further.

Pharmacological agents, such as bisacodyl, could be used as an adjunct in the assessment of colonic motility in these patients. The use of bisacodyl instillation into the colon to provoke high-amplitude contractions has been described[226, 230]. This technique could be applied to per-appendicostomy colonic manometry and extended to test different colonic regions as this technique allows simultaneous assessment of the whole colon. In addition, this technique could be used to investigate the abnormalities in colonic motility that are seen in children with Hirschsprung disease and anorectal malformations.

As in the case of the *in vitro* studies, it is clearly not possible, for ethical reasons, to study children with no gastro-intestinal motility problems and so the use of true controls is impossible. While per-appendicostomy catheter insertion provides a useful mechanism for investigating children with STC, not all children with STC require this procedure to be undertaken. As such, it is likely that retrograde catheter insertion using colonoscopy is likely to be required to investigate other children with colonic dysmotility in the clinical setting.

Percutaneous sacral nerve stimulation is an interesting area of research that is developing in relation to the management of children with urinary and/or faecal soiling. Stimulation methods previously described include using either an anal probe electrode or using a needle inserted in the area of the anterior tibial nerve[281, 282]. A less invasive alternative of using surface electrodes has been reported in a pilot study[283]. The mechanism of action of sacral nerve stimulation is currently unknown. However, improvements in urinary symptoms, such as daytime incontinence, urgency and bladder capacity, have been reported[281-283]. Similarly, sacral nerve stimulation has been used in the treatment of faecal soiling in children by inserting needle electrodes into the sacral nerve foramina (S2, 3 and 4)[284].

A study applying the surface electrode technique to children with slow-transit constipation and faecal soiling is being conducted at the Royal Children's Hospital, Melbourne. Preliminary results indicate that children may benefit from a definite, although occasionally temporary, improvement (Professor J. Hutson, personal communication). This is clearly a promising area of research and further studies, ideally prospective, randomised and blinded, are required to evaluate the efficacy of

this treatment modality. The mechanism of action of this therapy is unknown. It may be possible to undertake per-appendicostomy 24-hour colonic manometry before, during, and after electrical stimulation therapy to determine if there are any changes in colonic motility.

This series of studies has shown that a proportion of children with 'idiopathic' chronic constipation (in whom Hirschsprung disease has been excluded) have demonstrable abnormalities on scintigraphy, in the *in vitro* characteristics of colonic circular muscle neuromuscular transmission and on 24-hour *in vivo* studies of colonic pressure characteristics. It is likely that the symptoms displayed by this subgroup of severely affected children are not merely a reflection of behavioural disturbances or learned responses to painful defaecation. It is currently unclear what happens to children with chronic constipation once they reach adulthood, but some data on this subject is emerging. A recent longitudinal study in which children with constipation were followed into adolescence[285] reported that symptoms persisted in one third of patients. That some children with intractable constipation may not 'grow out of it' does support the concept that there may be an underlying physiological abnormality causing colonic dysmotility.

The final point to be made in relation to this population of children is that of terminology. The diagnostic criteria for intestinal neuronal dysplasia and hypoganglionosis are controversial and further confusion may have arisen by attempts to revise or extend their definitions[172]. In addition, the functional correlation of these proposed histological findings remains unknown. Until the functional and anatomical defects in the circuitry of the enteric nervous system in these children have been

further unravelled, then it could be argued that 'slow-transit constipation' is a more useful diagnostic term that reflects demonstrable radiological findings.

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10.1 Appendix 1:

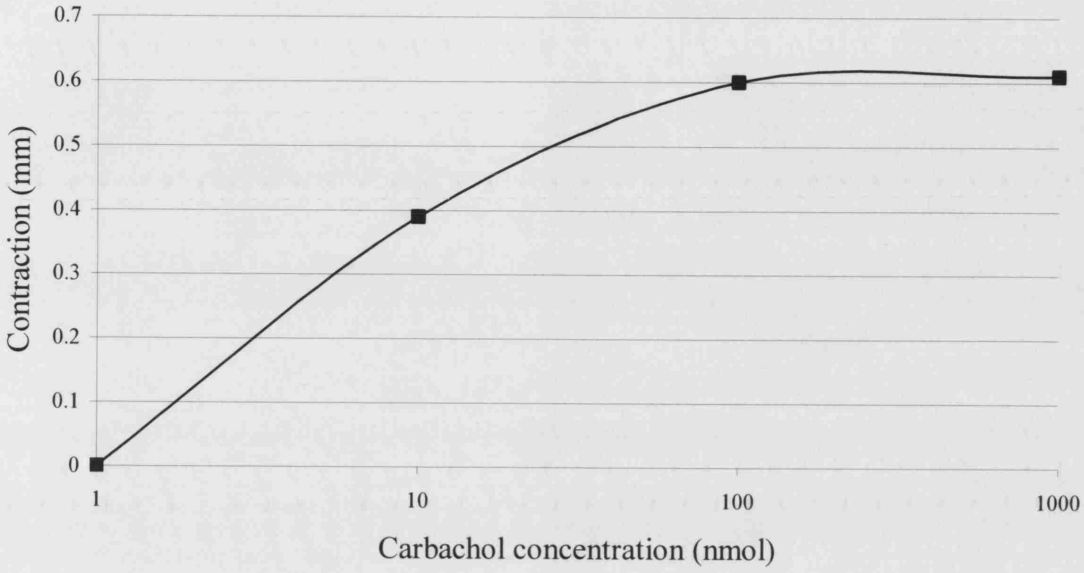

Figure 16 A: Example concentration/effect curve for carbachol for transverse colonic circular muscle from child with slow-transit constipation. Aliquots of carbachol (of increasing concentrations) were added sequentially to the organ bath, with 3 x 5 min washouts performed between each addition. Contractions (mm) are shown with respect to concentration of carbachol (nm). 1000 nmol (10 μmol) carbachol induces maximal contraction in this specimen.

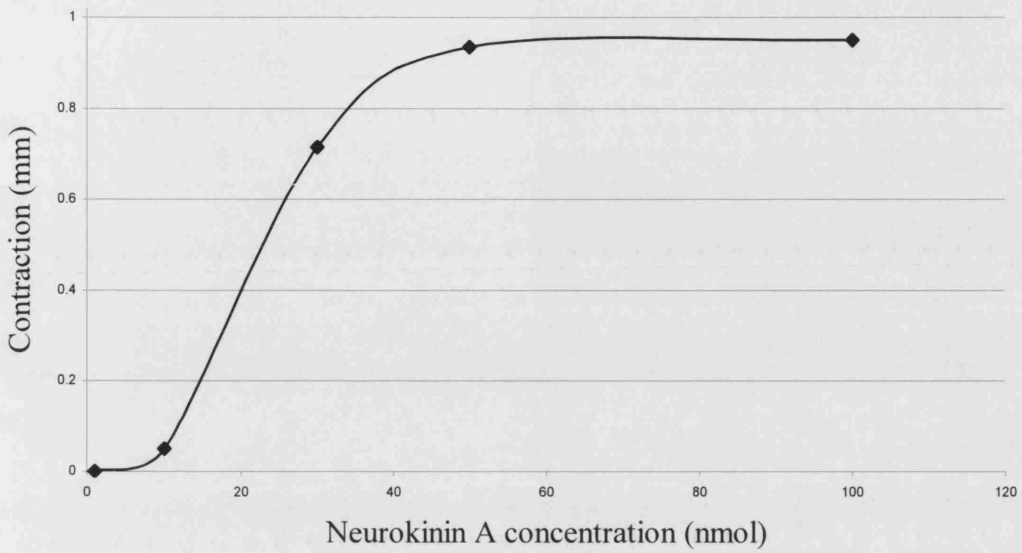

Figure 16 B: Example concentration/effect curve for neurokinin A (NKA) for adult transverse colonic circular muscle. Aliquots of NKA (of increasing concentrations) were added sequentially to the organ bath, with 3 x 5 min washouts performed between each addition. Contractions (mm) are shown with respect to concentration of NKA (nm). 100 nmol NKA induces maximal contraction in this specimen.

10.2 Appendix 2:

Immunohistochemistry for substance P (tachykinin, TK):

These immunohistochemistry described here was carried out by Dr. Bridget Southwell and Mrs Pamela Farmer. Acquisition of confocal images was undertaken by Dr. Bridget Southwell, Mrs Pamela Farmer and myself. Data analysis and figure preparation was performed by Dr. Bridget Southwell. This section is included in this appendix as it describes how the hospital pathologist's grading of SP neve fibre density was supported by confocal microscopy and counting of SP-IR profiles.

Biopsies were fixed in Zamboni's fixative (2% formaldehyde in 0.1 M phosphate buffer, 15% picric acid - pH 7.0) overnight, dehydrated in dimethylsulphoxide (DMSO) 3 x 10 minutes, washed in phosphate buffered saline (PBS) 3 x 10 minutes, then placed in 50% sucrose-OCT overnight and finally into OCT and frozen (as described previously[172, 173]). Cryostat sections (10 μM) were cut with circular muscle in cross section. Sections were incubated with normal sheep serum (10%, 30 minutes) followed by a rabbit antibody against SP (1:50, overnight, Zymed, San Francisco, California, USA) followed by a sheep anti-rabbit-FITC (1:200, 2 hours, Silenus/Chemicon International, California, USA). Sections were washed for 3 x 10 minutes in PBS, stained with 1% Chicago blue for 30 seconds, washed again (3 x 10 minutes) and mounted Mowiol. Sections were examined by the hospital pathologist and scored for SP density.

The samples used for physiology were not stained. Biopsies from adjacent regions of colon were fixed, stained and examined by the hospital pathologist and scored as normal, low (-2), or very low (-3) (Figure 17).

In order to determine if the pathologist grading represented different groups or a continuum, samples from 48 STC patients were separately examined to quantify SP density. Images were taken using a BioRad 1024 confocal laser scanning system mounted on a Zeiss Axioplan II microscope using excitation at 488 nm and 520/32 band pass filter. The number of SP-immunoreactive (SP-IR) profiles was counted by placing a grid (0.1 mm^2) on images of cross sections of transverse colon. The density of SP profiles is shown in Figure 18. The number of SP profiles was significantly different between STC normal and STC –3 groups, $p<0.001$ (Figure 18 A) (Krukal Wallace test – nonparametric ANOVA- sum ranks comparing median values and Dunn's multiple comparison test). While there was overlap in the range of each group (Figure 18 B), the number of SP profiles in the STC colons was not normally distributed but showed two peaks in distribution (Figure 18 C); one peak for less than 50 profiles and a second peak for more than 50 profiles. The number of profiles was also counted in normal colon removed 20 adults with carcinoma. The STC-normal group had similar number of profiles to the adult group and to the normal region of colon removed from 5 children with Hirschsprung disease.

As these results show, the pathologist's grading was able to distinguish between the high and low SP groups; for physiology results, the hospital pathologist definition was used. Children graded as –2 or –3 were designated as 'low SP' and the rest as 'normal SP'.

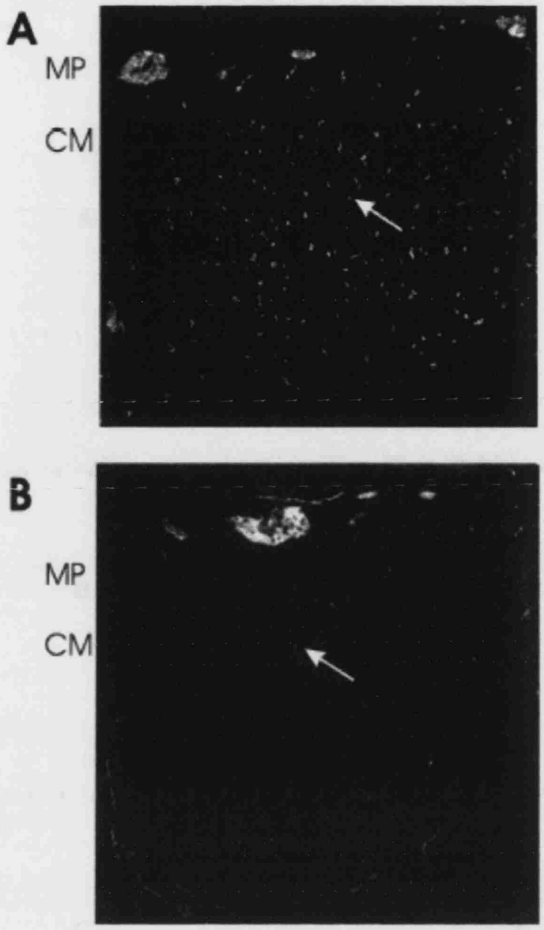

Figure 17: Tachykinin-immunoreactivity in colonic circular muscle of children with slow-transit constipation.

Density of TK-IR in CCM of children with STC was diagnosed as normal or low by the hospital pathologist. MP – myenteric plexus; CM – circular muscle.

A) An example of normal density of nerve fibres with TK-IR in transverse section of CCM from a child with STC. Immunoreactive nerve fibres are present in high numbers in the circular muscle and myenteric ganglia.

B) An example of low density of nerve fibres with TK-IR in circular muscle from another child with STC. Immunoreactive nerve fibres are sparse in the circular muscle, but visible in the myenteric plexus.

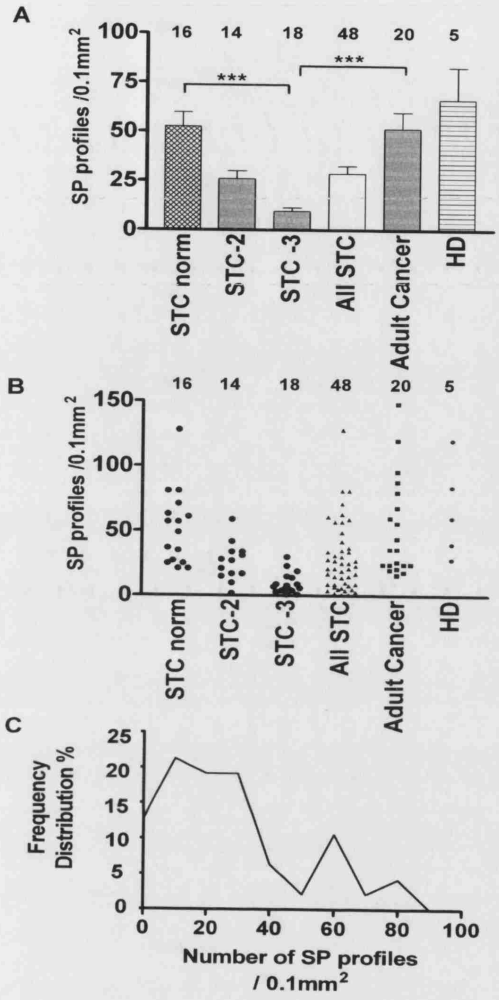

Figure 18: Density of SP-immunoreactive profiles in colonic circular muscle.

A) Mean (SE), colon from children with STC graded by hospital pathologist as having normal density for SP (STC norm), reduced SP density (STC-2) or very low SP density (SP-3). Adult cancer is the normal margin of colon resected for carcinoma in adults. HD is the normal margin of colon removed for Hirschsprung Disease in children. Numbers (n) are shown above columns. (B) Individual data, (C) Frequency of distribution of SP density in colon from 48 STC children. *** = $p < 0.001$.